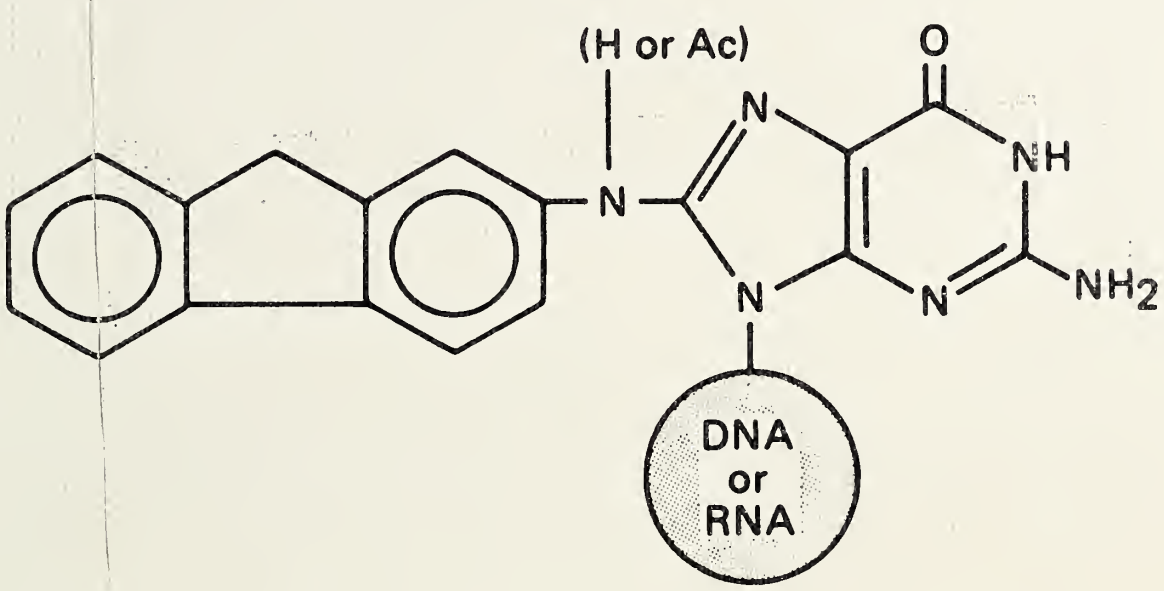
National Cancer Institute Monograph

Carcinogenic and Mutagenic N-Substituted Aryl Compounds



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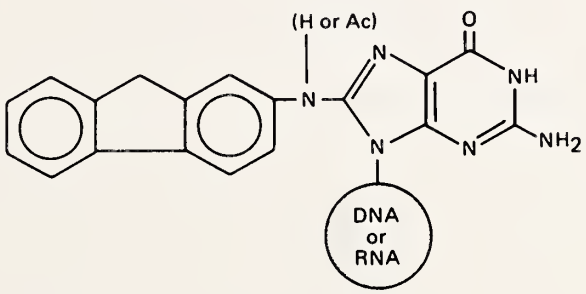
CARCINOGENIC AND MUTAGENIC N-SUBSTITUTED ARYL COMPOUNDS



Proceedings

of an

International Conference

held in

Rockville, Maryland

November 7–9, 1979

Sponsored by

Division of Cancer Cause and Prevention
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Organizing Committee

Snorri S. Thorgeirsson
Elizabeth K. Weisburger
Charles M. King
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Preface

Since the discovery of the so-called "aniline cancer" among factory workers in the German dye industry by Dr. Rehn in 1895, numerous aromatic amines and amides have been found to be carcinogenic in animal tests. A few compounds of this class are also known or suspected human carcinogens. Due to the extensive use of aromatic amines and compounds derived therefrom in the preparation of drugs, dyestuffs, pesticides, and polymers, a potential hazardous condition may exist for those employed in the production of the above-mentioned products. Moreover, recent data on the potential genotoxic hazard upon exposure to certain drugs and cosmetics containing aromatic amines and amides further emphasize the need for better understanding of the public health aspects as well as the mechanism by which these compounds cause their toxic effects.

Because some aromatic amine derivatives, such as 2-fluorenylacetamide and related compounds, have been extensively used as models in the study of the molecular events involved in the processes of chemical carcinogenesis, it was considered appropriate to organize a conference that would bring together scientists involved in both the public health and basic research aspects of the toxic effects of aromatic amines. Furthermore, 10 years had elapsed since the last exclusive conference on aromatic amines took place, and as witnessed by this volume, many new and exciting discoveries have surfaced in this research area during that time.

Arrangements for an international conference with attendees from Australia, Europe, Japan, and North America were made, and the Conference was held November 7–9, 1979, at the Sheraton-Potomac Hotel, Rockville, Maryland.

Elizabeth K. Weisburger, Ph.D.
Snorri S. Thorgeirsson, M.D.

N-Substituted Aryl Compounds in Carcinogenesis and Mutagenesis[1]

Elizabeth K. Weisburger[2, 3]

ABSTRACT—A brief history of the development of synthetic dyes derived from aromatic amines is given and the later consequences on workers' health. Metabolic studies and carcinogenicity tests of aromatic amines showed that the differences in susceptibility of various species to the amines cannot always be explained on the basis of metabolic handling of amines. Tests for mutagenicity of aromatic amines in histidine-dependent strains of the bacterium, *Salmonella typhimurium,* are often useful as indicators of possible carcinogens. However, the mutagenicity tests gave a false positive result when compared with those from carcinogenicity studies of the same compounds.—Natl Cancer Inst Monogr 58: 1–7, 1981.

The history of research on the carcinogenic and mutagenic effects of arylamines and their derivatives has its origin in the attempts by William Henry Perkin to develop a synthetic substitute for quinine in treatment of malaria (*1*). Upon oxidation of impure aniline, which contained various toluidines, Perkin obtained a dark sticky mass. Examination showed that this material, which Perkin named mauveine, chiefly *N*-phenylphenosafranine and its homologs (text-fig. 1), was useful in dyeing silk and wool to a violet color, highly admired by the Victorians. Discovery of the diazonium reaction of aromatic amines by Griess a few years later increased the possible application of aromatic amines in dye production. The Kekule theory of the benzene ring structure in 1863 then provided a theoretical basis for the preparative reactions used by chemists working in the dye industry.

These discoveries and the continued research of Kekule, Griess, and Perkin served to increase the development and utilization of synthetic dyes. Factories were established in Germany, Switzerland, and Great Britain for the production of materials for this new industry.

About 25 to 30 years later came the first warning that exposure to dyestuffs or their intermediates represented a hazard to workers. In 1895, Ludwig Rehn, a surgeon in Frankfurt-am-Main, reported a cluster of bladder cancer cases, all in employees of the dye industry. Within a few years, similar reports came from other countries where dyestuff factories had been established.

Although epidemiologic and case studies implicated 2-NA and benzidine as the probable carcinogens, experimental confirmation did not come until Hueper and his associates (*2*) reported on induction of bladder cancer in dogs by repeated administration of 2-NA.

Not long thereafter, Wilson, DeEds, and Cox (*3*) reported that the acetyl amide derived from 2-FA was carcinogenic to rats. Contrary to the results with the other carcinogens then known, 2-FAA induced numerous types of tumors but generally not at the point of application. This made the effects of 2-FAA more closely akin to "spontaneous" tumors and stimulated a great deal of interest in its use as a research carcinogen. Thus although the initial intended use of 2-FAA as an insecticide never came to commercial development because of its carcinogenicity, 2-FAA has become a model carcinogenic aromatic amine used in numerous experiments (*4*).

The study of these 2 compounds, 2-NA, once a commercial material, and 2-FAA, the model with no commercial use, has practically been the lifework for different groups of investigators. Although benzidine also has commercial importance, less attention has been paid to the mechanism of action of this dyestuff intermediate than to the model 2-FAA. Nevertheless, the major metabolites of benzidine have been identified in mice, rats, guinea pigs, rabbits, and dogs. Most easily obtained was the 3-hydroxy derivative, which was excreted as such or as a sulfate conjugate. In addition, acetyl derivatives or N-glucuronides or N-sulfates were also identified. Interest in benzidine has been renewed (*5*) and in the azo dyes derived therefrom for several reasons. One is the metabolic reduction of such dyes back to benzidine, and another is the neoplastic potential of certain of these dyes (*6*), illustrated in text-figure 2.

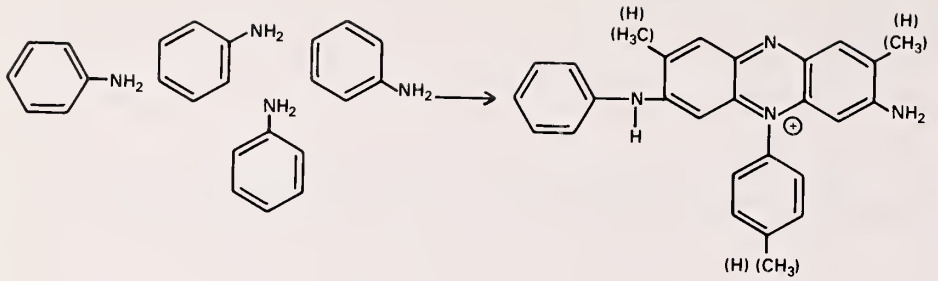
Of all aromatic amines, 2-FAA is the one researchers use most in model experiments to determine the mechanism of action of this type of chemical carcinogen. Metabolic studies followed by animal tests of the metabolites have shown that the numerous ring-hydroxylated and 9-hydroxylated derivatives of FAA are probably detoxification products. Metabolites derived from *N*-OH-2-FAA appear closer to the activated carcinogen on the basis of their reactivity and effects in animals (text-fig. 3). This information has led to extensive use by scientists of *N*-OH-2-FAA and especially *N*-AcO-2-FAA in various model experiments to determine the mechanisms of action of aromatic amine

Abbreviations: 2-NA = 2-naphthylamine; 2-FA = 2-fluorenamine; 2-FAA = *N*-fluorenylacetamide: OH = hydroxy; AcO = acetoxy.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

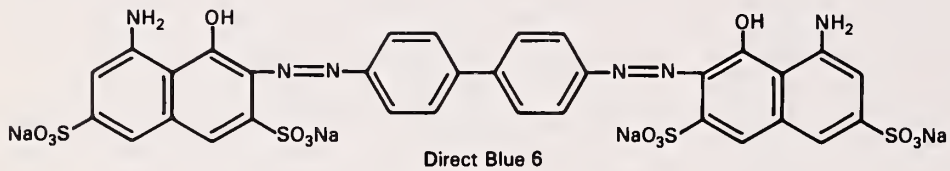
[2] Laboratory of Carcinogen Metabolism, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Bethesda, Maryland 20205.

[3] The secretarial assistance of Mrs. Frances Williams is greatly appreciated.



Mauveine

TEXT-FIGURE 1.—Structure of mauveine.



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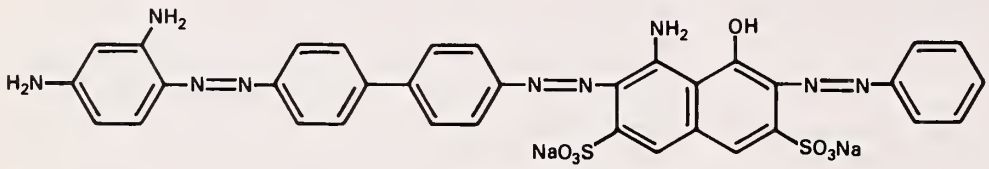

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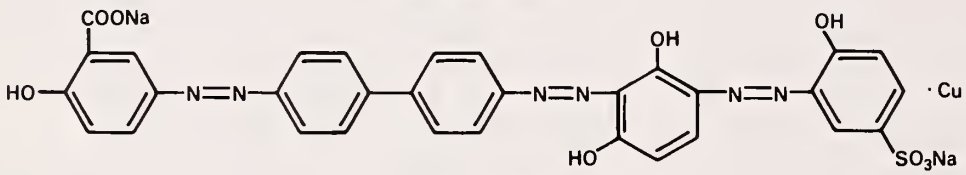
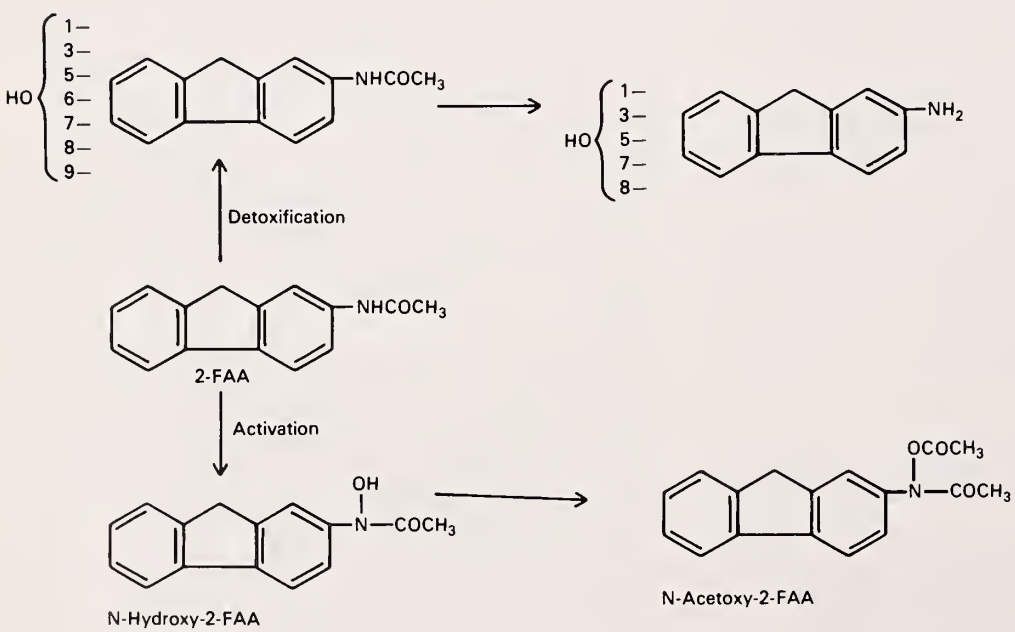


Direct Brown 95

TEXT-FIGURE 2.—Structures of 3 carcinogenic benzidine-based dyes.



N-Hydroxy-2-FAA

N-Acetoxy-2-FAA

TEXT-FIGURE 3.—Metabolic products of 2-FAA.

derivatives. Numerous studies on DNA structure and its function in various model systems or on DNA repair have used the acetoxy derivative as the activated carcinogen (*7*).

In vivo and in vitro metabolic studies on 2-NA have been accomplished largely by Boyland and associates and later by Radomski (*8*). These investigations demonstrated the diversity of the metabolites of 2-NA. Hydroxylation at the 1- and/or 6-positions with subsequent conjugation with sulfuric or glucosiduronic acid, acetylation–deacetylation, and the formation of N-sulfates or N-glucuronide, mercapturic acids, and a phosphate ester all served to yield the numerous metabolites identified. As with other aromatic amines, *N*-OH-2-NA represented an activated mutagenic metabolite (*7–9*).

Moreover, metabolic studies demonstrated the effects of the host on the agent. We are also interested in the opposite, i.e., the effect of the agent on the host. Aromatic amines affect various species and have some distinctive organotropic effects. As has been mentioned, the species in which the carcinogenicity of aromatic amines was first noted was man, who was and is exposed to a variety of these compounds in an occupational setting. Another primate, the monkey, has also proved susceptible to at least 1 aromatic amine, 2-NA (*8*), but not to another, 2-FAA (*10*). However, because of their scarcity, cost, and the long latent period, monkeys are used sparingly for research on the effects of aromatic amines.

Dogs were used by Hueper and associates (*2*) and are still valuable in certain instances because their response to many aromatic amines is analogous to that of man; cost and a long latent period are adverse factors. Of the other larger species that served as experimental models, rabbits often showed bladder tumors. For many reasons, cats have seldom been used for carcinogenicity studies. Guinea pigs seem to lack the enzyme system which N-hydroxylates the aromatic amides, but they may hydroxylate amines to a small extent. Hamsters offer some advantages because they are more likely to form bladder tumors than certain larger species. For most carcinogenicity studies, rats and mice appear to offer the best compromise between size, cost, and a response analogous to that of humans. Of course, there are many strains of these two species; each strain has special characteristics. Knowledge of these factors can aid in selection of the proper strain for any carcinogenicity study.

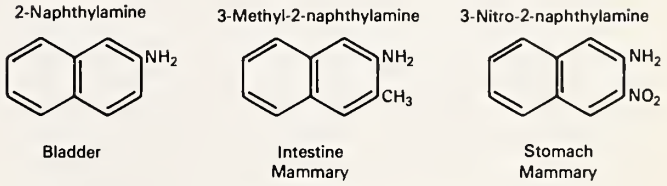
Other more unusual animal species scientists used to determine the response to the typical carcinogenic amide 2-FAA are fowl, guppies, *Mastomys,* rainbow trout, cotton rats, steppe lemmings, and certain newts (table 1).

Many aromatic amines or their derivatives have specific organotrophic effects in animal models; e.g., 2-NA generally yields bladder tumors in man, monkey, dog, and hamster. Substitution of an *o*-methyl in the 3-position of 2-NA yielded a mammary and intestinal carcinogen. However, a nitro group in the 3-position led to mammary and forestomach tumors as well as some lesions in the ear duct (*11*), as shown in text-figure 4. In male rats, 2-FAA induces liver cancer but mammary, bladder, or ear duct tumors were also observed. An analog, 2,7-diacetamidofluorene, had been for some time the only compound available that reliably induced carcinomas of the glandular stomach in rats

TABLE 1.—*Carcinogenic aromatic amines and species response*[a]

Species	Compounds			
	4-Amino-biphenyl	Benzidine	2-FAA	2-NA
Cat			+	
Chicken			+	
Cotton rat			0	
Dog	+	+	+	+
Guinea pig			0	+
Guppy			+	
Hamster		+	+	+
Human	+	+	?	+
Mastomys				
Monkey		0	? or 0	+
Mouse	+	+	+	+
X/Gf mouse			0	
Newt			+	
Rabbit	+		+	+
Rainbow trout			0	
Rat	+	+	+	+
Steppe lemming			0	

[a] + = active; 0 = not active; ? = unknown.

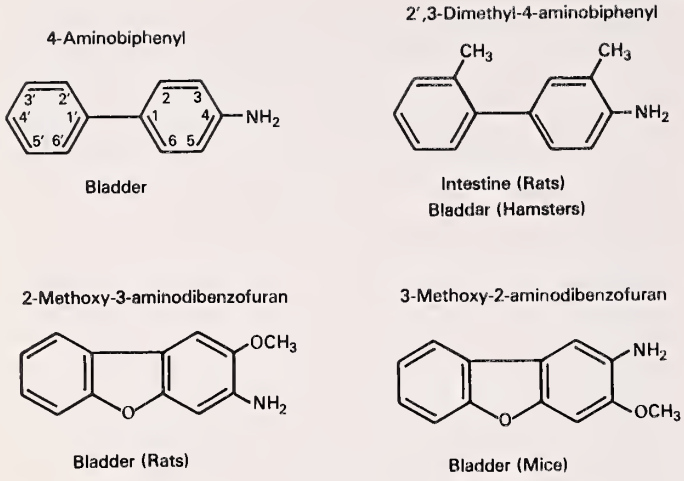


TEXT-FIGURE 4.—Structures of 2-NA and derivatives.

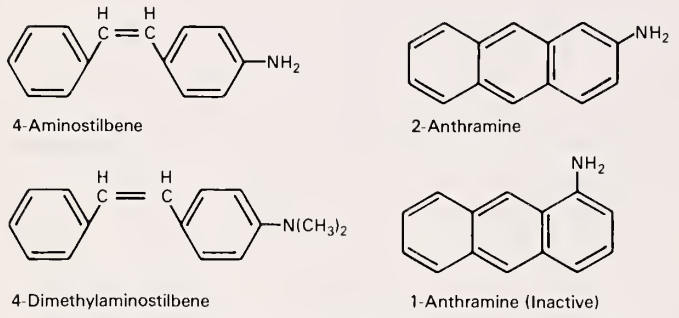
and *Mastomys* (*7*). Use of this compound was superseded by the discovery that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was more efficient in producing such tumors.

Although 4-aminobiphenyl is a potent bladder carcinogen in man or dogs, it had relatively little effect in rats. However, the addition of 2 methyl groups, as in 2',3-dimethyl-4-aminobiphenyl, led to an intestinal carcinogen for rats (text-fig. 5). Hamsters, on the other hand, responded with bladder tumors (*12*). Other compounds affecting the bladder in rats and mice, respectively, are 2-methoxy-3-aminodibenzofuran and the isomer 3-methoxy-2-aminodibenzofuran (*13*).

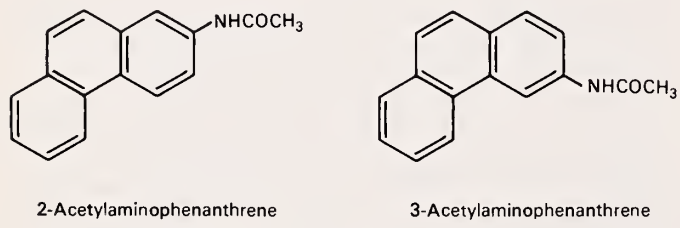
The skin or the sebaceous glands of the ear duct are the targets for 2-anthramine or aminostilbene derivatives, respectively. In both compounds, the amino group is adjacent to a conjugated system of alternating double and single bonds (text-fig. 6). Moreover, 2-anthramine was a potent mammary carcinogen in young female Sprague-Dawley rats, but the isomer 1-anthramine was ineffective (*14*). When fed to rats, the closely related 2-acetylaminophenanthrene led to leukemia, and ear duct, intestinal, and mammary tumors (text-fig. 7). However, changing the attachment of the acetamido group to the 3-position of phenanthrene led to ear duct and mammary tumors and no leukemia (*15*).



TEXT-FIGURE 5.—Structures of aminobiphenyl and aminodibenzofurans.

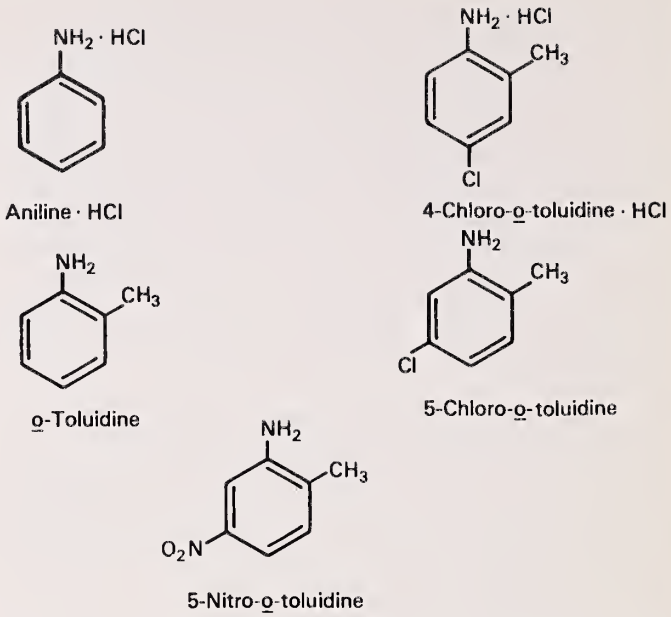


TEXT-FIGURE 6.—Aminostilbene and anthramine derivatives.



TEXT-FIGURE 7.—Acetylaminophenanthrenes.

More recent developments have shown that monocyclic aromatic amines or diamines may also be carcinogens, but this depends on the nature and position of the other substituents besides the amino group. Aniline hydrochloride induced hemangiosarcomas of the spleen in both male and female Fischer rats (*16*). Text-figure 8 depicts other compounds that caused hemangiosarcomas as *o*-toluidine (*17, 18*), 4-chloro-*o*-toluidine (*19*), 5-chloro-*o*-toluidine (*20*), and to some extent 5-nitro-*o*-toluidine (*21*). Thus the majority were *o*-toluidine derivatives. Both *m*- and *p*-cresidine and *o*-anisidine caused bladder tumors in rats; *p*-cresidine led to both bladder and liver tumors in mice (*22–24*). Surprisingly, as shown in text-figure 9, the phenol 4-amino-2-

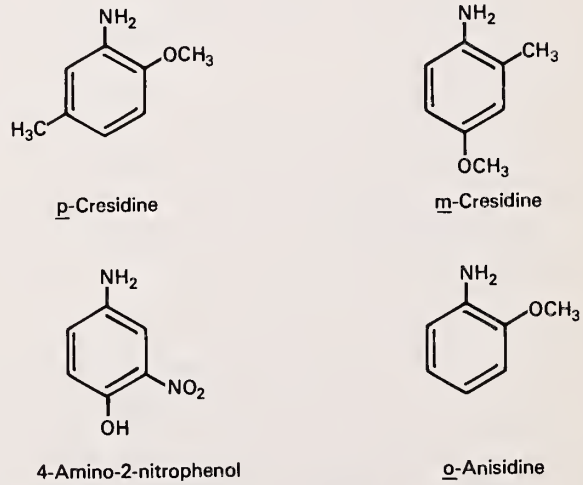


TEXT-FIGURE 8.—Monocyclic aromatic amines which cause hemangiosarcomas.

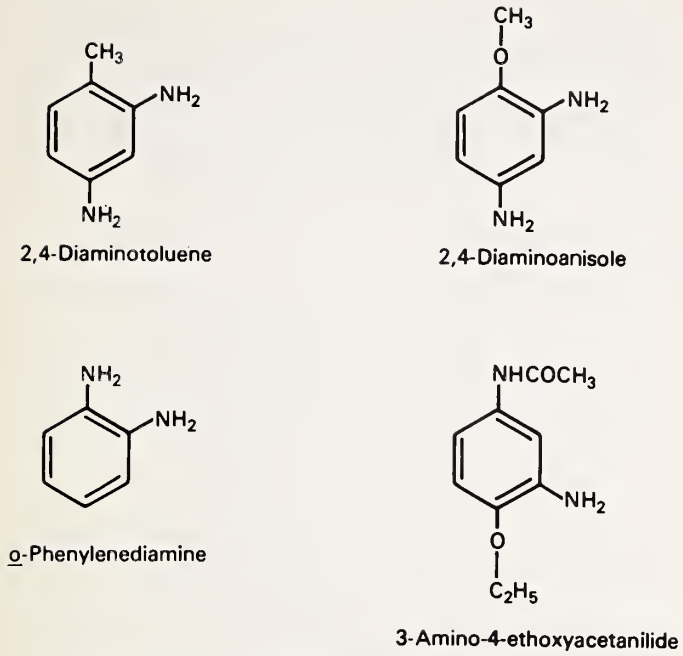
nitrophenol also produced bladder tumors in male rats (*25*).

Certain monocyclic diamines were carcinogens, including 2,4-diaminotoluene, *o*-phenylenediamine (*17, 26*), 2,4-diaminoanisole (*27*), and 3-amino-4-ethoxyacetanilide (*28*). Whereas the first 2 diamines named caused liver and other tumors, the latter 2, both containing ether linkages, affected the thyroid (text-fig. 10).

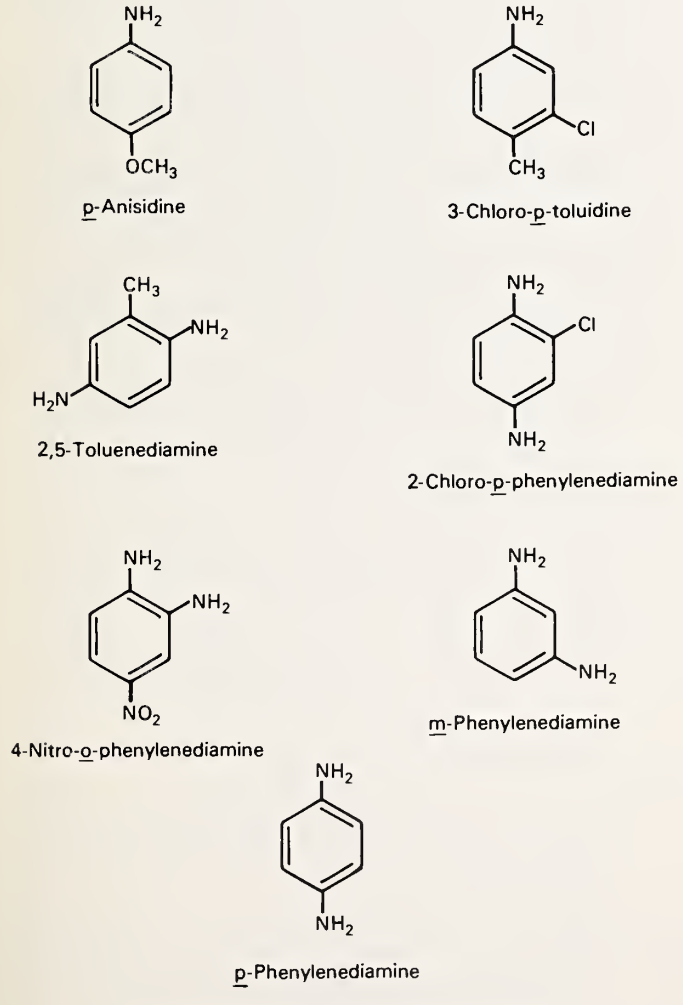
Among the many compounds (closely related to these active ones) that were inactive in tests conducted under similar protocols (text-fig. 11) are *p*-anisidine (*29*), 3-chloro-*p*-toluidine (*30*), *m*-phenylenediamine (*17*), and



TEXT-FIGURE 9.—Monocyclic aromatic amines which cause bladder tumors.



TEXT-FIGURE 10.—Carcinogenic monocyclic diamines.



TEXT-FIGURE 11.—Inactive compounds.

p-phenylenediamine and its 2-chloro-and 2-methyl analogs (*31–33*). Despite the carcinogenicity of *o*-phenylenediamine, its 4-nitro analog was inactive (*34*).

These studies indicate that the situation is complex, and the effects of the carcinogenic arylamines, both with respect to species, target organs, and types of tumors induced, vary greatly. Slight changes in structure can alter the effects dramatically. With most arylamines there are no metabolic or mechanistic studies that might furnish some explanation for the differences. Thus the need for research for solutions to these problems is continuous. Eventually, methods might be discovered to inhibit some of the activation pathways of arylamines.

However, these bioassays which have yielded the wealth of material on carcinogenicity of many amines are expensive and time-consuming. For practical and theoretical reasons, researchers have focused much attention on determining the effects of substances in bacteria or other simple organisms. Instead of tumors, the result would be mutations in bacteria or comparable changes in the genetic material of larger organisms. Such changes could be detected much earlier than the 2 years needed in an animal bioassay and at much less expense.

Initial studies on mutagenicity of aromatic amines and their derivatives, such as 2-FAA, in *Drosophila melanogaster* or *Neurospora crassa* were generally negative (*7*). However, the realization that 2-FAA required metabolic activation led to reexamination of its activated metabolites. Neither 2-FAA nor *N*-AcO-2-FAA induced point mutations in *Drosophila,* but the latter derivative caused small chromosome deletions, presumably mediated through the RNA-forming genes.

In *Bacillus subtilis,* various esters of *N*-OH-2-FAA, *N*-OH-4-acetylaminobiphenyl, *N*-OH-4-acetylaminostilbene, and *N*-OH-2-acetylaminophenanthrene all had mutagenic activity, as evidenced by inactivation of transforming DNA. The parent amines, amides, N-hydroxyamides, and N-hydroxyamines had little or no effect in the *Bacillus* system, which was an indication that the N-hydroxy esters were the active moieties.

Likewise, in *Escherichia coli* bacteriophage T_4, *N*-AcO-2-FAA and 7-flouro-*N*-AcO-2-FAA were mutagenic, but *N*-OH-1- and 2-NA or 2-FA were not (*35*). In other *E. coli* mutants, however, *N*-OH-1- and 2-NA were the only active compounds, whereas various other derivatives, both ring and N-hydroxylated, were totally inactive (*36, 37*). These data demonstrate the variability of the bacterial system.

Although other organisms have been proposed or used, the development of *S. typhimurium* as the indicator organism has probably facilitated the greatest number of investigations. These include correlations between carcinogenicity and mutagenicity of discrete chemicals and the use of mutagenicity in these bacteria as an indication of an activated metabolite. As a corollary, mutagenicity in bacteria has been used as an indicator in numerous model experiments on the mechanism of action of carcinogens (*38–40*).

The various strains of *Salmonella* currently used have been developed by Ames and associates (*41*). They are all histidine auxotrophs; when grown in media containing only a trace of histidine, only the cells which revert to histidine independence form colonies. These strains also have a

defective lipopolysaccharide coat which makes the bacterial cells more permeable to many molecules. Furthermore, the repair capabilities are lessened and sensitivity is increased (table 2).

Of all these bacterial strains, TA1538, which seems to be the most responsive to aromatic amines and closely related compounds (*42*), was used to identify the mutagenicity of hair dyes or their components (*43*) as shown in table 3.

Some discrepancies occur between the mutagenicity of some of these diamines and their effects in long-term studies. Generally, the mutagenicity tests were more likely to show false-positive than false-negative results. Other tests with various series of aromatic amines or their derivatives have shown a fairly good correlation between mutagenicity in bacteria and carcinogenicity in animals (*44–46*).

A new and interesting development in mutagenicity studies has been the "co-mutagenic" effect of 2-anthramine. This aromatic amine enhanced the mutagenicity of various bile acids (*47*) to a degree far above that of anthramine or the bile acids alone. Use of such a system may afford leads on the possible enhancement of deleterious effects when subjects are exposed to mixtures of environmental materials.

Therefore, if the deficiencies of the most commonly used mutagenicity test systems are kept in mind, these systems offer scientists a fast, convenient, and much less expensive tool to prescreen compounds for long-term studies. They are also of value in mechanistic and structure-activity research and in the follow-up of genetic differences in the activation of carcinogens. Use of mutagenicity to determine the possible interactive enhancement of deleterious effects of chemical mixtures offers new avenues for research efforts.

TABLE 2.—S. typhimurium *strains*

Designation	Gene	Mutation type detected
TA1535	*his* G	Base-pair substitution
TA1537	*his* C	Frameshift
TA1538	*his* D	Frameshift
TA98	*his* D	Frameshift
TA100	*his* G	Base-pair substitution

TABLE 3.—*Comparison of carcinogenicity and mutagenicity tests of aromatic diamines possibly used as hair dye components*

Compound	Carcinogenicity		Mutagenicity
	Rats	Mice	
2,4-Diaminoanisole	+	+	+
2,4-Toluenediamine	+	+	+
2,5-Toluenediamine	–	–	+
m-Phenylenediamine	–	–	+
4-Nitro-*o*-phenylenediamine	–	–	+
o-Phenylenediamine	+	+	+
2-Nitro-*p*-phenylenediamine	–	+ (Female only)	+
p-Phenylenediamine	–	–	–

One can be sure that, back in 1856, William Henry Perkin did not realize or foresee all the benefits, the harm, and all the other implications his research on dyes and their intermediates would bring. However, his efforts afforded a more colorful world for all and a challenging one for those scientists interested in aryl amines and the mechanisms of their actions.

REFERENCES

(*1*) SCOTT TS: Carcinogenic and Chronic Toxic Hazards of Aromatic Amines. Amsterdam: Elsevier, 1962

(*2*) HUEPER WC, WILEY FH, WOLFE HD: Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. J Ind Hyg Toxicol 20:46–84, 1938

(*3*) WILSON RH, DEEDS F, COX AJ JR: The toxicity and carcinogenic activity of 2-acetaminofluorene. Cancer Res 1:595–608, 1941

(*4*) WEISBURGER EK, WEISBURGER JH: Chemistry, carcinogenicity, and metabolism of 2-fluorenamine and related compounds. Adv Cancer Res 5:331–431, 1958

(*5*) MORTON KC, KING CM, BAETCKE KP: Metabolism of benzidine to *N*-hydroxy-*N*,*N*'-diacetylbenzidine and subsequent nucleic acid binding and mutagenicity. Cancer Res 39:3107–3113, 1979

(*6*) Carcinogenesis Testing Program, National Cancer Institute: 13-Week subchronic toxicity studies of Direct Blue 6, Direct Black 38, and Direct Brown 95 dyes. Carcinogenesis Tech Rep No. 108. Washington, D.C.: U.S. Govt Print Off, 1978

(*7*) WEISBURGER EK: Laboratory chemicals. *N*-2-Fluorenylacetamide and derivatives. *In* Carcinogens in Industry and the Environment (Sontag JM, ed). New York: Marcel Dekker, 1981, pp 583–666

(*8*) RADOMSKI JL: The primary aromatic amines: Their biological properties and structure-activity relationships. Annu Rev Pharmacol Toxicol 19:129–157, 1979

(*9*) WEISBURGER JH, WEISBURGER EK: Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(*10*) THORGEIRSSON SS, SAKAI S, ADAMSON RH: Induction of monooxygenases in rhesus monkeys by 3-methylcholanthrene: Metabolism and mutagenic activation of *N*-2-acetylaminofluorene and benzo[*a*]pyrene. J Natl Cancer Inst 60:365–369, 1978

(*11*) HADIDIAN Z, FREDRICKSON TN, WEISBURGER EK, et al: Tests for chemical carcinogens. Report on the activity of derivatives of aromatic amines, nitrosamines, quinolines, nitroalkanes, amides, epoxides, aziridines, and purine antimetabolites. J Natl Cancer Inst 41:985–1036, 1968

(*12*) SO BT, WYNDER EL: Induction of hamster tumors of the urinary bladder by 3,2'-dimethyl-4-aminobiphenyl. J Natl Cancer Inst 48:1733–1738, 1972

(*13*) CLAYSON DB, COOPER EH: Cancer of the urinary tract. Adv Cancer Res 13:271–381, 1970

(*14*) GRISWOLD DP JR, CASEY AE, WEISBURGER EK, et al: The carcinogenicity of multiple intragastric doses of aromatic and heterocyclic nitro or amino derivatives in young female Sprague-Dawley rats. Cancer Res 28: 924–933, 1968

(*15*) MILLER JA, SANDIN RB, MILLER EC, et al: The carcinogenicity of compounds related to 2-acetylaminofluorene. II. Variations in the bridges and the 2-substituent. Cancer Res 15:188–199, 1955

(*16*) Carcinogenesis Testing Program, National Cancer Institute: Bioassay of aniline hydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 130. Washington, D.C.: U.S. Govt Print Off, 1978

(*17*) WEISBURGER EK, RUSSFIELD AB, HOMBURGER F, et al: Testing of 21 environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. J Environ Pathol Toxicol 2:325–356, 1978

(*18*) Carcinogenesis Testing Program, National Cancer Institute: Bioassay of *o*-toluidine hydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 153. Washington, D.C.: U.S. Govt Print Off, 1979

(*19*) _____: Bioassay of 4-chloro-*p*-toluidine hydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 165. Washington, D.C.: U.S. Govt Print Off, 1979

(*20*) _____: Bioassay of 5-chloro-*o*-toluidine for possible carcinogenicity. Carcinogenesis Tech Rep No. 187. Washington, D.C.: U.S. Govt Print Off, 1979

(*21*) _____: Bioassay of 5-nitro-*o*-toluidine for possible carcinogenicity. Carcinogenesis Tech Rep No. 107. Washington, D.C.: U.S. Govt Print Off, 1978

(*22*) _____: Bioassay of *m*-cresidine for possible carcinogenicity. Carcinogenesis Tech Rep No. 105. Washington, D.C.: U.S. Govt Print Off, 1978

(*23*) _____: Bioassay of *p*-cresidine for possible carcinogenicity. Carcinogenesis Tech Rep No. 142. Washington, D.C.: U.S. Govt Print Off, 1979

(*24*) _____: Bioassay of *o*-anisidine hydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 89. Washington, D.C.: U.S. Govt Print Off, 1978

(*25*) _____: Bioassay of 4-amino-2-nitrophenol for possible carcinogenicity. Carcinogenesis Tech Rep No. 94. Washington, D.C.: U.S. Govt Print Off, 1978

(*26*) _____: Bioassay of 2,4-diaminotoluene for possible carcinogenicity. Carcinogenesis Tech Rep No. 162. Washington, D.C.: U.S. Govt Print Off, 1979

(*27*) _____: Bioassay of 2,4-diaminoanisole sulfate for possible carcinogenicity. Carcinogenesis Tech Rep No. 84. Washington, D.C.: U.S. Govt Print Off, 1978

(*28*) _____: Bioassay of 3-amino-4-ethoxyacetanilide for possible carcinogenicity. Carcinogenesis Tech Rep No. 112. Washington, D.C.: U.S. Govt Print Off, 1978

(*29*) _____: Bioassay of *p*-anisidine hydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 116. Washington, D.C.: U.S. Govt Print Off, 1978

(*30*) _____: Bioassay of 3-chloro-*p*-toluidine for possible carcinogenicity. Carcinogenesis Tech Rep No. 145. Washington, D.C.: U.S. Govt Print Off, 1978

(*31*) _____: Bioassay of *p*-phenylenediamine dihydrochloride for possible carcinogenicity. Carcinogenesis Tech Rep No. 174. Washington, D.C.: U.S. Govt Print Off, 1979

(*32*) _____: Bioassay of 2-chloro-*p*-phenylenediamine sulfate for possible carcinogenicity. Carcinogenesis Tech Rep No. 113. Washington, D.C.: U.S. Govt Print Off, 1978

(*33*) _____: Bioassay of 2,5-toluenediamine sulfate for possible carcinogenicity. Carcinogenesis Tech Rep No. 126. Washington, D.C.: U.S. Govt Print Off, 1978

(*34*) _____: Bioassay of 4-nitro-*o*-phenylenediamine for possible carcinogenicity. Carcinogenesis Tech Rep No. 180. Washington, D.C.: U.S. Govt Print Off, 1979

(*35*) CORBETT TH, HEIDELBERGER C, DOVE WF: Determination of the mutagenic activity to bacteriophage T4 of carcinogenic and noncarcinogenic compounds. Mol Pharmacol 6:667–679, 1970

(*36*) PEREZ G, RADOMSKI JL: The mutagenicity of the N-hydroxynaphthylamines in relation to their carcinogenicity. Ind Med Surg 34:714–716, 1965

(*37*) MUKAI F, TROLL W: The mutagenicity and initiating activity of some aromatic amine metabolites. Ann NY Acad Sci 163:828–836, 1969

(*38*) JACOBS M, MATNEY TS, GRIFFIN AC: Inhibitory effects of selenium on the mutagenicity of 2-acetylaminofluorene (AAF) and AAF derivatives. Cancer Lett 2:319–322, 1977

(*39*) ROSIN MP, STICH HF: Inhibitory effect of reducing agents on *N*-acetoxy- and *N*-hydroxy-2-acetylaminofluorene-induced mutagenesis. Cancer Res 38:1307–1310, 1978

(*40*) SAKAI S, REINHOLD CE, WIRTH PJ, et al: Mechanism of in vitro mutagenic activation and covalent binding of *N*-hydroxy-2-acetylaminofluorene in isolated liver nuclei from rat and mouse. Cancer Res 38:2058–2067, 1978

(*41*) AMES BN, LEE FD, DURSTON WE: An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci USA 70:782–786, 1973

(*42*) MCCANN J, CHOI E, YAMASAKI E, et al: Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. Proc Natl Acad Sci USA 72:5135–5139, 1975

(*43*) AMES BN, KAMMEN HO, YAMASAKI E: Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. Proc Natl Acad Sci USA 72:2423–2427, 1975

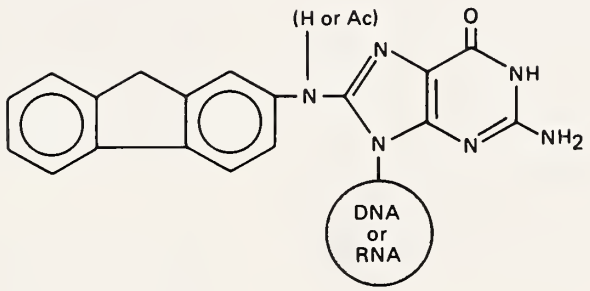
(*44*) BROWN MM, WASSOM JS, MALLING HV, et al: Literature survey of bacterial, fungal, and *Drosophila* assay systems used in the evaluation of selected chemical compounds for mutagenic activity. J Natl Cancer Inst 62:841–871, 1979

(*45*) POIRIER LA, DE SERRES FJ: Initial National Cancer Institute studies on mutagenesis as a prescreen for chemical carcinogens: An appraisal. J Natl Cancer Inst 62:919–926, 1979

(*46*) MCMAHON RE, CLINE JC, THOMPSON CZ: Assay of 855 test chemicals in ten tester strains using a new modification of the Ames test for bacterial mutagens. Cancer Res 39:682–693, 1979

(*47*) SILVERMAN SJ, ANDREWS AW: Bile acids: Co-mutagenic activity in the *Salmonella*–mammalian-microsome mutagenicity test. J Natl Cancer Inst 59:1557–1559, 1977

Session I: Human Exposure



Session Chairman: Bailus Walker, Jr.

Discussion Chairman: Takashi Sugimura

Occupational Exposure to Aromatic Amines: Benzidine and Benzidine-Based Dyes[1]

Bailus Walker, Jr.,[2] *and* Abbie Gerber[2, 3]

ABSTRACT—Large-scale production of aromatic amines that serve as intermediates in the aniline dye industry began in this country during World War I. In the United States, the first known occupationally induced aromatic amine cancers of the bladder were observed at a dye factory in 1931. Additional cases of benzidine-related bladder cancer were subsequently reported at this same facility and at numerous other dye works in this country and throughout the world. In 1974, the Occupational Safety and Health Administration promulgated a standard to control the production and use of benzidine in the workplace. Although hazards associated with benzidine exposure have been reduced, a carcinogenic risk due to benzidine-based dye exposure is now apparent. In addition, several dyes produced from *o*-tolidine and *o*-dianisidine, carcinogenic compounds structurally related to benzidine, have demonstrated metabolism to their respective parent compounds. The history of occupational bladder cancer in the United States is traced and measures taken by the Occupational Safety and Health Administration to reduce exposure to this hazard are described.—Natl Cancer Inst Monogr 58: 11-13, 1981.

Large-scale production of aromatic amines that serve in this country as intermediates in the aniline dye industry began during World War I (*1*). In 1931, the first occupational aromatic amine cancers of the bladder were observed among workers at a factory of a major producer. Three years later, Gehrmann reported the discovery of 27 cases of bladder cancer among those working with aromatic amines at this company's installation. In 1937, the physician at this plant's dye works recorded a total of 83 cases of bladder cancer, 56 of which occurred during the previous 3 years. This company did not withdraw benzidine from commercial dye production until 1973 (*2*). According to its records, 339 employees with urinary bladder cancer were granted compensation between 1956 and 1974.

In 1952, Barsotti and Vigliani (*3*) reported that workers in an Italian factory who were exposed solely to benzidine experienced a 22% frequency of bladder papilloma. A more contemporary (1974) accounting of this bladder cancer experience was given by Maltoni (*4*), who reported that of 1,449 workers exposed in the dyestuff industries, 189 cases of bladder and upper urinary tract tumors were detected.

Thirteen years after the Italians reported the carcinogenic hazards of benzidine and other azo dyes, Goldwater et al. (*5*) of the New York State Department of Labor revealed similar findings at a chemical plant in Buffalo that produced aromatic amines and azo dyes. Among 366 workers exposed to benzidine, α-naphthylamine, and β-naphthylamine, 96 had bladder cancer. More specifically, among workers exposed solely to benzidine, 17 (21%) of 76 developed bladder cancer.

Studying workers who were exposed to benzidine and β-naphthylamine in a Cincinnati dye plant, Mancuso and Coulter (*6*) found 6 cases of bladder cancer as opposed to the 0.33 expected. By 1966, the number of cases of bladder and kidney cancers among employees of this company had risen to 42 (*7*).

Zavon et al. (*8*) reported on the mortality experience of 25 workers who terminated employment when the same Cincinnati chemical plant ceased manufacturing benzidine in the late 1950's. By 1970, clearly 13 (52%) of 25 employees had already developed bladder cancer. A total accounting of the incidence of bladder cancer following employment in aromatic amine or azo dye production has yet to be undertaken in the United States.

It was against this background that two major courses of action were pursued during the period from 1972 to 1974. One consisted of an industry-wide investigation of current industrial hygiene and engineering control practices for the production and use of benzidine and the assessment of its attending bladder cancer risk. Investigations indicated a lack of closed system technology and widespread potential of contamination due to benzidine sulfate.

The second course of action was the promulgation of the first public health measure in the United States that addressed control of the production of aromatic amines, including benzidine, in the industrial setting (*9*). This standard excludes from regulation any products, including dyes, that contain less than 1,000 ppm benzidine.

What do we know about the hazards associated with dye products made from benzidine? In 1971, an epidemiologic study revealed that of 200 male patients with bladder cancer, 8.5% worked in the dye industry compared with 1.4% in the matched control group (*10*); benzidine-based dyes

Abbreviation: OSHA = Occupational Safety and Health Administration.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7-9, 1979.

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[3] *Present address:* Chronic Chemical Hazards Program, Health Sciences, Consumer Product Safety Commission, Bethesda, Maryland 20207.

were confirmed to have been among the dyes used by these workers. For most of these 17 workers with bladder cancer, the primary routes of exposure were probably through skin absorption and inhalation, but another route of exposure was through ingestion for 3 of them, who were kimono painters with a habit of licking the tips of their paintbrushes.

In 1975, Rinde and Troll (*11*) demonstrated that in the monkey 4 benzidine-based dyes were metabolized to benzidine and its metabolite, monoacetyl benzidine. Three years later, Korosteleva (*12*) detected benzidine in the sera and tissues of rats fed C.I. Direct Red 13, another benzidine-based dye.

In the same year, metabolism studies of 3 benzidine-based dyes in rats and mice were conducted at the National Cancer Institute (*13*). Both rats and mice metabolized the dyes to benzidine. In this same 13-week subchronic study, a significant excess of hepatocellular carcinomas and hepatic neoplastic nodules was demonstrated in rats. The hepatocellular carcinomas appeared 5 weeks after initial exposure. This time-to-tumor interval is the shortest ever reported in the Institute's Bioassay Program (*14*).

Consistent with experimental study results, Boeniger stated that benzidine was detected in the urine of workers who had contact with various benzidine-based dyes used in different manufacturing processes under present industrial working conditions (*14*). None of the samples of the dyes contained greater than 25 ppm benzidine.

Recently, Matthews (*15*) found that in a study of the metabolic conversion of 6 additional benzidine-based dyes in mongrel female dogs, every benzidine-based dye administered resulted in a quantifiable excretion of this compound in the urine.

In response to the promulgation of the benzidine standard, the only two manufacturers of the chemical in the United States ceased production in 1976, which eliminated our domestic sources for dye production (*16*). Currently, only one manufacturer is producing benzidine-based dyes in this country.

On the basis of results of its bioassay studies, the National Cancer Institute cooperated with the National Institute for Occupational Safety and Health and issued a "Current Intelligence Bulletin" in April 1978 (*17*) to inform the industrial health community and workers of the potential carcinogenic risk to employees exposed to the benzidine-based dyes: Direct Black 38, Direct Brown 95, and Direct Blue 6.

Two compounds closely related to benzidine, *o*-tolidine (dimethylbenzidine) and *o*-dianisidine (dimethoxybenzidine), are also used in the production of dyes. Estimates of the production and importation of these dyes by major companies (at least 7 are excluded) are shown in table 1.

Thousands of workers in industries, such as those involved in the production of dyes, textiles, paper, and leather goods are potentially exposed to dyes based on benzidine, *o*-tolidine, and *o*-dianisidine. More than one of these dyes may be used concurrently in the same industry.

TABLE 1.—*Production and imports of dyes made from benzidine, o-tolidine, and o-dianisidine, 1978* [a]

Dye base	Pounds (presscake basis)
Benzidine	1–2 million
o-Tolidine	800,000
o-Dianisidine	1,329,000

[a] Dyes Environmental and Toxicology Organization, Inc.: Personal communication.

This use of multiple dyes makes the enumeration of the number of workers exposed and the extent of exposure to any specific dye more difficult.

The textile dyeing and finishing industry employed an estimated 71,400 production workers in 1976; of these, about 7% were potentially exposed to these dyes (*18*). In 1979, the leather tanning and finishing industry had 20,400 production workers, of which 1,000 spray machine operators or colorists may have had some exposure to the dyes (*19, 20*). Also, in that same year, the National Institute of Occupational Safety and Health estimated that about 79,200 workers in 63 occupations were potentially exposed to benzidine-based dyes (*21*). Exposure to these compounds may occur by ingestion, skin absorption, and inhalation (*21–23*). No definitive studies that establish the primary route of exposure have been conducted.

Many of these dyes may be complex mixtures and difficult to analyze; they may also contain impurities that are carcinogenic (*14*). For example, 4-aminobiphenyl, an OSHA-regulated carcinogen, and 2,4-diaminoazobenzene, a carcinogen documented as such by the International Agency for Research on Cancer, were found as impurities in a batch of Direct Black 38 (*24*).

The magnitude and potential severity of worker exposure are grave enough that we believe appropriate regulatory action by OSHA is warranted. The National Institute of Occupational Safety and Health and OSHA have developed a "Health Hazard Alert," which summarizes the biologic effects of these dyes and recommends actions that employers should take on a voluntary basis to reduce exposure to these potential human carcinogens by their employees.

The "Health Hazard Alert" has been distributed through an extensive network. Copies have been sent to OSHA's 10 regional offices and 92 area offices. The Institute developed a mailing list of about 8,000 persons and organizations active in occupational health to whom a mailing has been made. Important industrial producers and consumers have been identified, and copies have been sent to each of them. Concerned individual unions, the American Federation of Labor–Congress of Industrial Organizations, and its Industrial Union Department have also been notified. Each of the 24 OSHA State Plan States has received a copy.[4]

Other interested Federal agencies were informed directly or through groups such as the Interagency Regulatory Liaison Group and the National Toxicology Program. The public and the news media received announcements from OSHA's Office of Public Affairs. Additional copies have been disseminated through a large system of informal contacts by OSHA scientists. Professional societies, such as

[4] "State Plan States" are those which have chosen local administration of OSHA regulations in place of Federal enforcement; OSHA maintains an oversight role.

the American Occupational Medical Association, American Public Health Association, American Industrial Hygiene Association, and the American Medical Association, have been sent copies. Trade associations have been notified as well.

In addition to the Alert, OSHA has issued to all compliance officers a field directive pertaining to the 3 benzidine-based dyes that induced cancer in experimental animals (*13*). This document provides guidance necessary for effective environmental control and for regulatory action when adequate control measures for these dyes are not met.

As we move forward with our efforts to decrease worker exposure to these dyes and other hazardous substances, we welcome additional scientific and epidemiologic information which can ultimately be translated into regulatory action for control of those agents which may adversely affect the health of workers.

REFERENCES

(*1*) Hueper W: Occupational and Environmental Cancers of the Urinary System, New Haven: Yale Univ Press, 1969

(*2*) Castleman B: Du Pont's Record in Business Ethics: Another View. The Washington Post, July 15, 1979

(*3*) Barsotti M, Vigliani E: Bladder lesions from aromatic amines. Arch Industrial Hyg Occup Med 5:234–241, 1952

(*4*) Maltoni C: Precursor lesions in exposed populations as indicators of occupational cancer risk. Ann NY Acad Sci 271:444–447,1976

(*5*) Goldwater L, Rosso A, Kleinfeld M: Bladder tumors in a coal tar dye plant. Arch Environ Health 11:814–817, 1965

(*6*) Mancuso T, Coulter E: Methods of studying the relation of employment and long-term illness-cohort analysis. Am J Public Health 49:1525–1536, 1959

(*7*) Mancuso T, El-Attar A: Cohort study of workers exposed to beta-naphthylamine and benzidine. J Occup Med 9:277–285, 1967

(*8*) Zavon M, Hoegg U, Bingham E: Benzidine exposure as a cause of bladder tumors. Arch Environ Health 27:1–7, 1973

(*9*) Carcinogens. Fed Register 39 (Part III): 3755–3797, 1974 (January 29, 1974)

(*10*) Yoshida O, Harada T, Miyagawa M, et al.: Bladder cancer in workers of the dyeing industry. Igaku No Ayumi 79:426–422, 1971

(*11*) Rinde E, Troll W: Metabolic reduction of benzidine azo dyes to benzidine in the rhesus monkey. J Natl Cancer Inst 55:181–187, 1975

(*12*) Korosteleva T, Skachkov A, Kondrat'yeva A: Blastomogenic action of aniline dyes and determination of carcinogens in tissues. Gig Tr Prof Zabol 10:22–26, 1978

(*13*) Carcinogenesis Testing Program, National Cancer Institute: 13-Week subchronic toxicity studies of Direct Blue 6, Direct Black 38, and Direct Brown 95 dyes. Carcinogenesis Tech Rep No. 108. Washington, D.C.: U.S. Govt Print Off, 1978

(*14*) National Institute for Occupational Safety and Health, Center for Disease Control: Special hazard review of benzidine-based dyes, DHHS (NIOSH) Publ No. 80–109. Cincinnati, Ohio: National Institute for Occupational Safety and Health, 1980

(*15*) Matthews H: Metabolism studies in the dog: Benzidine congener dyes (a preliminary report from the National Toxicology Program). Presented at the 46th Meeting of the Interagency Collaborative Group on Environmental Carcinogenesis, Washington, D.C., 1979

(*16*) Environmental Protection Agency: Survey of the manufacture, import, and uses for benzidine-related substances, and related dyes and pigments (JRB Associates Report: EPA–560/13-79-005). Washington, D.C., April 1, 1979

(*17*) National Institute for Occupational Safety and Health: Current Intelligence Bulletin No. 24, Direct Blue 6, Direct Black 38, Direct Brown 95. DHEW (NIOSH) Publ No. 78–148. Cincinnati, Ohio: National Institute for Occupational Safety and Health, 1978

(*18*) U.S. Bureau of Labor Statistics: Industry Wage Survey: Textile Dyeing and Finishing, June 1976–1977. Washington, D.C.: U.S. Govt Print Off, 1978

(*19*) _____: Employment and Earnings, vol 26. Washington, D.C.: U.S. Govt Print Off, 1979

(*20*) _____: Industry Wage Survey: Leather Tanning and Finishing. Bull No. 1835. Washington, D.C.: U.S. Govt Print Off, 1973

(*21*) Meigs J, Brown R, Sciarini L: A study of exposure to benzidine and substituted benzidines in a chemical plant. Arch Industrial Hyg Occup Med 4:533–540, 1951

(*22*) Meigs J, Sciarini L, Sandt W: Skin penetration by diamines of the benzidine group. Arch Industrial Hyg Occup Med 9:122–132, 1954

(*23*) International Business Machines Corp: Report to Environmental Protection Agency on dermal absorption of Direct Black 38 in rabbits. Submitted to OSHA, September 1979

(*24*) International Agency for Research on Cancer Working Group: Some Aromatic Azo Compounds. IARC Monogr: Evaluation of Carcinogenic Risk of Chemicals to Man, vol 8. Lyon: IARC, 1975, pp 91, 151, 267

Specific Aromatic Amines as Occupational Bladder Carcinogens[1]

David B. Clayson[2]

ABSTRACT—The effect of specific aromatic amines in inducing bladder cancer among industrial workers exposed to these chemicals is documented. Most occupational bladder tumors are recognized to be due to 4 chemicals: 4-aminobiphenyl, 2-naphthylamine, benzidine, and commercial 1-naphythylamine that is contaminated with the 2-isomer. The consequences of this exposure are discussed.—Natl Cancer Inst Monogr 58: 15–19, 1981.

Three factors facilitate the discovery of an occupational cancer: 1) A readily definable population should be adequately exposed to the noxious agent; 2) the cancer(s) induced should not be of high frequency in the control or nonexposed population; and 3) a thinking and astute clinician must observe and draw attention to the incidence of cancer in the exposed population.

Occupational bladder cancer possesses each of these attributes. Workers in chemical factories often received excessive exposures to the noxious agents; e.g., men engaged in flaking freshly distilled 2-NA were continuously exposed to vast quantities of this agent, and nearly all developed bladder cancer *(1)*. In the unexposed population, bladder cancer is infrequent. Thus in 1965, of 145,000 male and 123,000 female white U.S. citizens who died of cancer, 5,200 and 2,400 (3.6 and 2.0%), respectively, had had cancer of the urinary bladder *(2)*. Similar figures could be presented for other years or other westernized nations. Such mortality data were sufficiently low that the German urologist, Rehn *(3)*, in 1895, considered worthy of reporting the fact that 3 patients with bladder cancer who worked in the same factory and another also engaged in manufacturing magenta (fuschin) from aniline in another factory presented at his clinic. Rehn surmized these cancers were associated with occupation and called the disease "aniline cancer," although later it was discovered *(4)* that aniline was not the causative agent.

Many more case reports concerning occupational bladder cancer appeared during the next half century. These were comprehensively surveyed by the late Dr. Wilhelm C. Hueper (*5*). To medical officers in factories, it appeared that the following were the most likely agents to be associated with occupational bladder cancer: 2-NA, 1-NA, benzidine, and aniline; however, in as complex a system as the chemical industry, something more than frequent case reports was required

IDENTIFICATION OF SPECIFIC AGENTS

The Association of British Chemical Manufacturers decided to investigate occupational bladder cancer in depth in the late 1930's, but the investigation was delayed for 10 years by World War II. In 1947, Case et al. (*6*) started a thorough epidemiologic investigation of the British chemical industry. From all cooperating factories, they obtained records that provided data on each man employed and the job performed. In the study population were 4,622 men who had worked in the industry for more than 6 months between 1921 and 1950. From the United Kingdom registry of death certificates, 127 of these men were recorded as having died from cancer of the bladder. If this population had not been exposed to noxious agents, 3–5 cases would have been expected to have developed; i.e., working in the cooperating factories led to a 30- to 40-fold increase in the risk of developing bladder cancer.

Because this type of cancer often has a long prognosis, patients may die while the disease is in remission, and the fact that they at one time had clinically diagnosed bladder cancer may not be recorded on their death certificates. Case and his colleagues (*6*), therefore, attempted to determine how many instances of the disease had occurred. They talked to industrial medical officers, examined the records of neighboring hospitals, read press reports of coroner's inquests, and interviewed workers and relatives of the men who had died. By these contacts, 262 cases of bladder cancer in the work force were determined. They had intended to compare this morbidity data with the bladder cancer morbidity of the male workers of Birmingham, England, a city without a major chemical manufacturing industry. However, this attempt had to be abandoned when they discovered an area in this city with an unexpectedly high bladder cancer morbidity (*7*).

Combination of the employment histories of the workers with the mortality and morbidity data enabled Case to determine the risk of bladder cancer development in those working with one or more of the suspect chemicals (table 1). Aniline showed a barely statistically significant excess of bladder cancer. When men employed in the production of auramine and magenta from aniline were excluded, the excess risk disappeared. In a separate survey the manufacture, but not the purification, of these two

Abbreviations: 1- or 2-NA = 1- or 2-naphthylamine; 4-BPA = biphenylamine.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 42d and Dewey Streets, Omaha, Nebraska 68105. *Present address*: Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada.

TABLE 1.—*Morbidity and mortality from occupational exposure to aromatic amines in the British chemical industry from 1921–50* [a]

Agent	Bladder cancer			
	Cases	Deaths	Deaths expected	Latency to death, yr [b]
Aniline	6	3	0.35	—
Aniline without dyes [c]	4	1	0.54	—
2-NA [d]	55	25	0.30	18
Benzidine [d]	34	10	0.75	18
1-NA [d]	19	6	0.70	22
Mixed exposure	144	81	1.48	—

[a] *See (4, 6).*
[b] Dashes indicate too few cases for determination.
[c] Dyes were auramine and magenta.
[d] Data of those exposed to amine and aniline are included.

TABLE 2.—*Response of different species to occupational bladder carcinogens*

Species	Tumor site		
	2-NA	Benzidine	4-BPA
Man	Bladder	Bladder [a]	Bladder
Dog	"	"	"
Rabbit	None	Not tested	"
Rat	Bladder	Liver, intestine, ear duct	Breast, intestine
Mouse	Liver	Liver	Liver, bladder
Hamster	Bladder [b]	"	

[a] Three bladder cancers were in 8 to 10-yr-old dogs.
[b] 2-NA comprised 1% of the diet.

dyes indicated an excess bladder cancer risk (*4*). Whether auramine and magenta were the carcinogens, or, e.g., substances, such as Milcher's ketone which might have been formed as intermediates or impurities during manufacture, caused the cancers is still an open question (*4*).

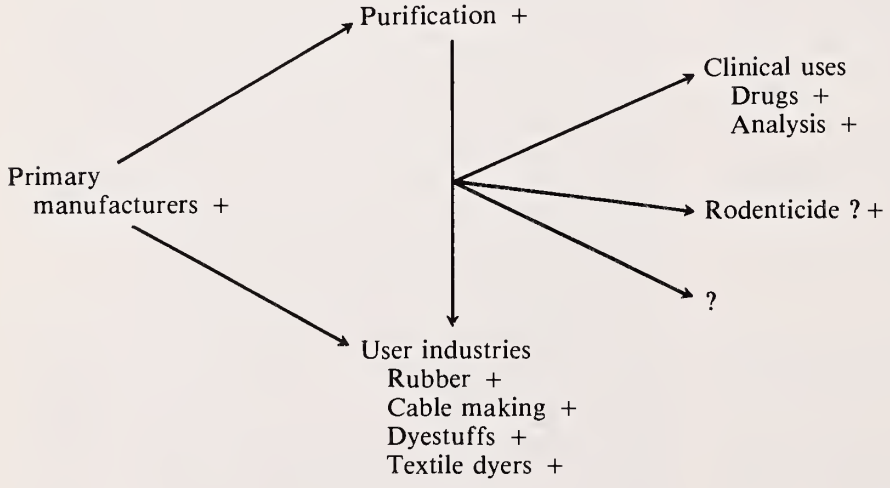
Exclusion of aniline as a contributor to the excess of bladder cancer enabled Case et al. (*6*) to add men exposed to aniline and one other suspect aromatic amine to those exposed to that agent only. In this way, it was possible to show that 2-NA was the worst offender in the induction of bladder cancer, benzidine was the next, and 1-NA was the least offensive. As made by the British chemical industry at that time, 1-NA contained substantial amounts of 2-NA, i.e., from 4 to 10%, (*8*) and it is still debatable whether the 1-NA or the potently carcinogenic impurity within it was responsible for the tumors. Men exposed to 2-NA for as short a period as 6–12 months developed tumors, whereas with the 1-isomer, exposure for 5 or more years was necessary. Case's pioneer investigation has been confirmed by other studies (*9–11*). The cooperation of the industry was not always forthcoming, as evidenced by a report from France (*12*): One of the investigators was fired for demonstrating that benzidine was a potent human bladder carcinogen.

Shortly after the publications of Case et al. (*4, 7*), Melick and his co-workers (*13, 14*) reported that 4-BPA was exceptionally effective in inducing 53 bladder cancers in 315 men (16%) exposed to it in one plant.

This is the limit of our present knowledge of aromatic amines as occupational bladder carcinogens. No new occupational bladder carcinogens have been established since 1955. Aromatic amines induce cancer in many animal tissues; however in man, only bladder cancer has been adequately investigated among workers in a few industries (table 2); this gap in knowledge should be filled.

RESULTS OF USE OF CARCINOGENIC AROMATIC AMINES

The chemical industry usually makes chemicals to sell. We would expect to find an excess of bladder cancer among those involved in the use of the proved noxious agents (text-fig. 1). Case's attempt to use Birmingham, England, as a control area for his morbidity studies of workers in the chemical industry who had cancer of the bladder provided the first example. A large rubber manufacturer used, as a rubber compounding ingredient, a



TEXT-FIGURE 1.—Spread of occupational bladder carcinogens throughout the environment (experience in the United Kingdom). *Plus sign* indicates bladder cancer was reported.

complex of 1- and 2-NA with acetaldehyde (Nonox S) and the result of workers' exposure to this complex or the amines contained within it was an excess mortality from bladder cancer. Management officials of the rubber industry were not as cooperative as those of the chemical industry, and the epidemiologic techniques were not as refined. As far as I have ascertained, this compounding ingredient was not used in the United States, nor has the incidence of bladder tumors been excessive among workers in the manufacture of rubber (*15, 16*). Davies (*17*) subsequently showed that among British electric cable makers who used rubber containing Nonox S, the rate of bladder tumors was also high. The use of 1-naphthylthiocyanate as a rodenticide (*18*), benzidine in medical practice for the detection of occult blood in feces or urine (*19*), 2-NA mustard in therapy for polycythemia [table 3; (*20*)], and benzidine-based dyes in kimono painting (*21*) are all examples of actual or possible effects of the dispersion of the noxious agents from the primary manufacturer to other environments. If industries were to obtain adequate capital, I am sure it would be possible to manufacture these aromatic amines safely, but I doubt that their many uses could be as effectively safeguarded.

The extent of occupational bladder cancer has been examined by a limited number of investigations in which the occupational history of patients with bladder cancer was compared with those of controls matched for sex, age, residence, smoking history, and other factors (*19, 22–25*). Thus Anthony and Thomas in Leeds, England (*19*), Wynder et al. in New York (*22, 23*), Dunham and associates in New Orleans (*24*), and Cole and co-workers in Boston (*25*) have conducted such studies on large numbers of patients. Overall, their results indicate that perhaps 20–30% of all bladder cancer affecting males living in industrialized areas is of an occupational etiology (*19*) and cast suspicion on specific local industries. However, great caution should be exercised in the acceptance of such results because of the limited numbers of people from a specific industry that are represented in the bladder cancer and control groups. Furthermore, these studies only rarely point to the same industries being affected as different chemical intermediates are used in each locality, e.g., the use of Nonox S as a rubber compounding ingredient in the United Kingdom but not in the United States.

Anthony and Thomas (*19*) drew attention to tailors' cutters and hairdressers who possibly developed bladder cancer at an earlier than average age and also to workers in the dye and textile industries, and perhaps electrical cable workers who were at excess risk. Cole et al. (*25*) inculpated the rubber and leather industries; such findings should not be considered as definite evidence for a carcinogenic risk in a given industry but rather as a strong indication that comprehensive epidemiologic and chemical use surveys need to be made. Unfortunately, such investigations are still far too infrequent (*26, 27*).

Removal of the major bladder cancer hazards, mainly 2-NA and benzidine, from the British rubber industry in 1949 presented an opportunity for epidemiologists to determine whether this action was effective in reducing the risk of workmen contracting bladder cancer. Fox et al. (*28*) assembled the names and employment records of all workers in the rubber industry in the United Kingdom, excluding those who were born elsewhere. They arranged with the Registrar General of the United Kingdom to receive copies of death certificates when any of the 40,000 men in the survey died. With the use of computer techniques, they studied causes of death of any kind in this population and compared them with those in the general population. Whether removal of the noxious aromatic amines in 1949 reduced the mortality of bladder cancer to the level of that in the general population was the major question. The latest report I have seen on this study (*29*) was inconclusive because inadequate numbers of cases of bladder cancer had been accumulated in the rubber industry group for one to calculate whether any degree of excess risk remains. This in itself is possibly encouraging. Completion of this study will provide a convincing commentary on the identification of a cancer hazard, government and industry cooperation in its removal, and demonstration that the remedial action was effective. Documentation of other hazards in a similar way is highly desirable.

TABLE 3.—*Bladder cancer after use of chlornaphazin (2-NA mustard) to treat polycythemia*

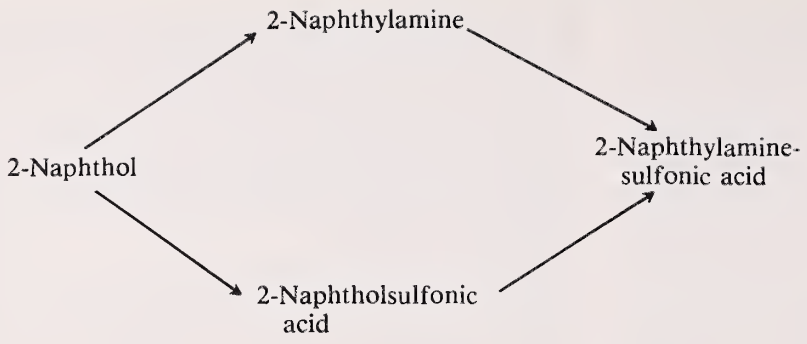
Chemical or group	Total dose of chlornaphazin, g	Total No. of patients	Patients with bladder cancer	
			No.[a]	Percent
Chlornaphazin	>200	5	4	80
	50–200	25	6	24
	1–50	31	3	10
Total	1–>200	61	13	21
Controls	None	40	0	0
$^{31}PO_4$	None	46	0	0

[a] Eight other patients had abnormal urinary cytology.

CONSEQUENCES OF THE OCCUPATIONAL DISEASE

The fact that agents that induce bladder carcinoma have been and sometimes are still being used in the work environment presents difficult problems. Knowing that they have been proved to be carcinogenic to humans, we must ask if their continued use is justified in any but the most exceptional circumstances. A population of workers have been exposed to these agents and are at high risk from bladder cancer; they need protection from the results of their exposure. There is another group of workers who have already developed cancer of the bladder who must receive adequate compensation for themselves or their dependents. I am most familiar with the United Kingdom's actions in these areas and will, therefore, discuss them rather than the less adequate response in the United States.

As soon as the rubber industry in the United Kingdom realized the consequences of the use of Nonox S and the other ingredients containing bladder carcinogens, they relinquished the use of these chemicals. In 1953, the chemical industry gave up the use of 2-NA and replaced all essential processes involving this chemical. For example, 2-naphthylaminesulfonic acids were formerly made by con-



TEXT-FIGURE 2.—Alternate routes to 2-naphthylamine-sulfonic acid production.

TABLE 4.—*United Kingdom Statutory Instrument: Carcinogenic substances (aromatic amines) Order No. 897*

Prohibited substances	Controlled substances
Benzidine	1-NA (containing less than 1% of the 2-isomer)
2-NA	*o*-Dianisidine
4-BPA	3,3'-Dichlorobenzidine
4-Nitrobiphenyl	*o*-Tolidine
Salts of the above	Auramine
	Magenta
	Mixtures containing less than 1% prohibited substances

version of 2-naphthol to 2-NA by the Bucherer reaction first and then sulfonation of the amine. Sulfonation of 2-napthol followed by amidation provided a safer alternative in which 2-NA was not deliberately produced (text-fig. 2). In 1967, with the full agreement of the chemical industry, the British Government introduced a Statutory Order (*30*), which prohibited the manufacture of the most hazardous bladder carcinogens and strictly controlled certain of their analogs (table 4). I was told that, by the mid 1970's, producers of chemicals found alternates for every essential use of benzidine. Ironically, in view of recent National Cancer Institute bioassay results demonstrating the carcinogenic potency of some of these chemicals (*31*), the importation into the United States of benzidine-based dyes was still permitted because they contained less than 1% benzidine. One Italian chemical company has, I am informed, now produced noncarcinogenic alternates for these potentially harmful materials.

The previous use of these hazardous aromatic amines has left a population of working people at high risk from bladder cancer. The early detection of cancer and prompt treatment should help increase their chances of survival. Urinary cytology, the microscopic examination of urine sediments for malignant transitional cells, was introduced during the 1950's into the chemical and rubber industries in the United Kingdom (*32*). Physicians believed it was more effective than urinary cystoscopy because it was less traumatic and, consequently, far fewer of the population-at-risk defaulted from regular periodic examinations. The best description of the results of urinary cytology is Melamed and Koss' (*33, 34*) study of a U.S. population exposed to 4-BPA. Of 505 persons exposed to the compound, 435 had negative cytology, 59 positive cytology, and 9 were not definitely interpretable. Three of 435 persons developed 2 carcinomas and 1 papilloma, i,e., they were false negatives, whereas 35 of 59 with positive cytology developed histologically confirmed bladder carcinoma. Of the 24 patients who were apparent false positives, 10 were lost to follow-up, 7 died from other causes before tumors developed, and 1 remained cytologically positive. This technique is not perfect, but it certainly offers an opportunity for clinicians to select men of an exposed population, who are developing bladder cancer, with only a 10% failure rate. In those persons not exposed to harmful agents, the high rate of false positives is disadvantageous (*35, 36*). Other immunologic and biochemical approaches to this problem are needed (*37, 38*).

The workmen who, as a result of occupational exposure, develop bladder cancer certainly deserve compensation. Families deprived of their breadwinner are in even more desperate need of such compensation. At its simplest, English law states that if an employer willingly permits an employee to be exposed to an agent or process he knows or should know to be hazardous, he is liable for damages. In England, this approach has permitted workmen or their dependents to claim substantial damages in addition to workmen's compensation. When the mortality from the disease increased 30–40 times among workmen, there was little reason to doubt the source of exposure. However, removal of the noxious agents and the progressively less hazardous environment means that such claims for compensation will, I am sure, be more actively defended (*39*). Compensation of employees exposed to agents which only fractionally raise risk relative to the general population is much more difficult. Should industry or the community be expected to compensate all workers who develop cancer whether or not it is work related? Where should the line be drawn between compensation and no compensation?

CONCLUSIONS

Aromatic amine-induced bladder cancer is the story of what epidemiologic cancer research should and can do with full industrial and governmental cooperation. A few other human (as opposed to animal) carcinogens are known, among which are cigarette smoking (which is responsible for most lung cancer and a significant proportion of bladder cancer), asbestos, vinyl chloride, and estrogens. They could be approached in the same way as the study of and action on occupational bladder cancer in the United Kingdom. This investigation illustrates what

can be done if government, industry, and academia are willing to cooperate. Am I wrong in thinking that the slower progress in the United States illustrates the consequences of an adversary relationship?

REFERENCES

(1) Williams MH: Industrial bladder cancer. Preventative measures. Acta Un Int Contra Cancr 18:676–683, 1962

(2) Segi M, Kurihara M, Matsuyama T: Cancer mortality for selected sites in 24 countries. No. 5 (1964–1965). Sendai, Japan: Department of Public Health, Tohoku Univ School of Med, 1969

(3) Rehn L: Ueber Blasentumoren bei Fuchsinarbeitern. Arch Clin Chir 50:588, 1895

(4) Case RA, Pearson JT: Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part II. Further considerations of the role of aniline and of the manufacture of auramine and magenta (fuchsin) as possible causative agents. Br J Ind Med 11:213–216, 1954

(5) Heuper WC: Occupational Tumors and Allied Diseases. Springfield, Ill.: Charles C Thomas, 1942

(6) Case RA, Hosker ME, McDonald DB, et al: Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part I. The role of aniline, benzidine, alpha-naphthylamine and beta-naphthylamine. Br J Ind Med 11:75–104, 1954

(7) Case RA, Hosker ME: Tumour of the urinary bladder as an occupational disease in the rubber industry in England and Wales. Br J Prev Soc Med 8:39–50, 1954

(8) Butt LT, Strafford N: Papilloma of the bladder in the chemical industry. Analytical methods for the determination of benzidine and β-naphthylamine. Recommended by ABCM Subcommittee. J Appl Chem 6:525–539, 1956

(9) Goldwater LJ, Rosso AJ, Kleinfeld M: Bladder tumors in a coal tar dye plant. Arch Environ Health 11:814–817, 1965

(10) Mancuso TF, El-Attar AA: Cohort study of workers exposed to beta-naphthylamine and benzidine. J Occup Med 9:277, 1967

(11) Parkes HG: The epidemiology of the aromatic amine cancers. *In* Chemical Carcinogens. Am Chem Soc Monogr No. 173 (Searle CS, ed). Washington D.C.: Am Chem Soc, 1976, pp 462–480

(12) Desoille H, Hochfeld M, Aboulker P: Le dépistage des tumeurs vésicales chez les travailleurs des produits colorants. Premiers résultats de cystoscopie systèmatique. Arch Mal Prof 9:1497, 1948

(13) Melick WF, Escue HM, Naryka JJ, et al: The first reported cases of human bladder tumors due to a new carcinogen—xenylamine. J Urol 74:760–766, 1955

(14) Melick WF, Naryka JJ, Kelly RE: Bladder cancer due to exposure to para-aminobiphenyl: A 17-year follow-up. J Urol 106:220–226, 1971

(15) McMichael AJ, Spirtas R, Kupper II: An epidemiologic study of mortality within a cohort of rubber workers, 1964–1972. J Occup Med 16:458–464, 1974

(16) Monson RR, Fine LJ: Cancer mortality and morbidity among rubber workers. J Natl Cancer Inst 61:1047–1053, 1978

(17) Davies JM: Bladder tumours in the electric-cable industry. Lancet 2:143–146, 1965

(18) Annotation: A dangerous rodenticide. Lancet 2:1183, 1966

(19) Anthony HM, Thomas GM: Tumors of the urinary bladder: An analysis of the occupation of 1,030 patients in Leeds, England. J Natl Cancer Inst 45:879–895, 1970

(20) Thiede T, Christensen BC: Blaeretumoret inducerede af Klornafazinbehandling. Ugeskr Laeger 137:661–666, 1975

(21) Yoshida O, Miyakawa M: Etiology of bladder cancer. Metabolic aspects. *In* Analytic and Experimental Epidemiology of Cancer (Nakahara W, et al, eds). Baltimore: Univ Park Press, 1973, pp 31–39

(22) Wynder EL, Onderdonk J, Mantell N: An epidemiological investigation of cancer of the bladder. Cancer 16:1388–1407, 1963

(23) Wynder EL, Goldsmith R: The epidemiology of bladder cancer: A second look. Cancer 40:1246–1266, 1977

(24) Dunham LJ, Rabson AS, Stewart HL, et al: Rates, interview, and pathology study of cancer of the urinary bladder in New Orleans, Louisiana. J Natl Cancer Inst 41:683–709, 1968

(25) Cole P, Hoover R, Freidell GH: Occupation and cancer of the lower urinary tract. Cancer 29:1250–1260, 1972

(26) Decoufle P: Cancer risks associated with employment in the leather and leather products industry. Arch Environ Health 34:33–37, 1979

(27) Baxter PJ, White WG, Barnes GM: Bladder cancer in car workers. Lancet 1:377, 1977

(28) Fox AJ, Lindars DB, Owen R: A survey of occupational cancer in the rubber and cable-making industries: Results of five-year analysis. Br J Ind Med 31:140–151, 1974

(29) Fox AJ, White GC: Bladder cancer in rubber workers. Do screening and doctor's awareness distort the statistics? Lancet 1:1009–1011, 1976

(30) Statutory Instrument. Carcinogenic substances (aromatic amines): Order No. 897. London: HM Stat Off, 1967

(31) Carcinogenesis Testing Program, National Cancer Institute: 13-Week subchronic toxicity studies of Direct Blue No. 6, Direct Black No. 38, and Direct Brown No. 95 dyes. Carcinogenesis Tech Rep No. 108. DHEW Publ. No. NIH–78–13–58. Springfield, Va.: Natl Tech Inform Serv, 1978

(32) Crabbe JG, Cresdee WC, Scott TS, et al: Cytological diagnosis of bladder tumours amongst dyestuff workers. Br J Ind Med 13:270–276, 1956

(33) Melamed MM, Koss LG, Ricci A, et al.: Cytohistological observations in developing carcinoma of the urinary bladder in man. Cancer 13:67–74, 1960

(34) Koss LG, Myron R, Melamed MM, et al: Carcinogenesis in the human urinary bladder. Observations after exposure to para-aminobiphenyl. N Engl J Med 272:767–770, 1965

(35) Sarnaki CT, McCormack LT, Kiser WT, et al: Urinary cytology and the clinical diagnosis of urinary tract malignancy—A clinico-pathologic study of 1,400 patients. J Urol 106:761–764, 1974

(36) Frable WJ, Paxson L, Barksdale JA, et al: Current practice of urinary bladder cytology. Cancer Res 37:2800–2805, 1977

(37) Kumar S, Wilson R, Brenchley P, et al: Frequent elevation of tissue polypeptide antibody in the sera of workers exposed to bladder carcinogens. Int J Cancer 22:542–545, 1978

(38) Taylor G, Kumar S, Brenchley P, et al: Immunosurveillance in premalignant occupational bladder disease. Int J Cancer 33:478–493, 1979

(39) Davis JM, Somerville SM, Wallace DM: Occupational bladder tumor cases identified during ten years' interviewing of patients. Br J Urol 48:561–566, 1977

Studies on Mutagenic and Carcinogenic N-Substituted Aryl Compounds: Cosmetics and Drugs[1, 2]

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ABSTRACT—We aimed our studies toward gaining an understanding of some of the reactions and pathways involved in the metabolism and activation of the aromatic diamines used in hair dyes and of phenacetin used in analgesic mixtures. Comparison of the data obtained from human and animal tissues established that animal tissues can serve as suitable models for evaluation of the activity of these compounds in humans.—Natl Cancer Inst Monogr 58: 21–26, 1981.

Data from animal experiments are often necessary in the evaluation of health hazards involved in chemical exposures. One of the prerequisites for extrapolating research results from laboratory animals to humans is that the toxic compound in question is metabolized similarly in both organisms. If the toxicity seen in animals is caused by the formation of reactive intermediates during metabolism, it is imperative to know whether the same reactions will take place in humans before an estimation of risk can be undertaken. Many of the currently available short-term tests for predicting mutagenicity and carcinogenicity incorporate a metabolic activation step for detection of toxic effects. By comparing activities of human tissues with those of laboratory animals in such studies, we can learn whether experiments with animals can serve as useful models for the human situation.

Considerable human exposure occurs by ingestion of arylamine drugs and by uptake of arylamine cosmetics through the skin. Epidemiologic evidence suggests that heavy use of analgesic mixtures containing phenacetin is related to the development of transitional cell carcinoma of the renal pelvis (*1, 2*). However, the epidemiologic data for the aromatic diamine hair dye components still appear to be inconclusive with regard to possible human carcinogenicity associated with the use of hair dyes (3–5). On the other hand, several of the diamines have been shown to be carcinogenic in animal feeding tests (*6–8*). Investigations aimed at delineation of metabolic activation reactions of the diamine hair dye components and phenacetin when animal and human tissues are used should be helpful in the evaluation of data from these species that are related to carcinogenicity of these compounds. This report presents an overview of some of our studies concerning the mutagenicity and carcinogenicity of aromatic diamines and phenacetin.

STUDIES WITH AROMATIC DIAMINES

In the United States, where it was estimated that 40% of adult women were regular users (*9*), retail sales of hair dyes amounted to $250 million in 1971; oxidation or permanent hair dyes represent three-fourths of the money spent on hair dyes (*9*). These dyes are formed with a mixture of a primary, a coupler, and hydrogen peroxide. Each component penetrates the hair: The primary is oxidized by hydrogen peroxide, and the molecule formed reacts with a coupler to form an indodye which cannot escape from the hair (*10*). In black shades, 2,4-DAA is typically used as a coupler to give a blue coloration with the oxidized primary 1,4-diaminobenzene (also called "*p*-phenylenediamine").

The present concern regarding the human safety of hair dyes was initiated by the studies of Ames and his collaborators (*11*) when they showed that commercially available hair dyes contained compounds which could be detected as frameshift mutagens in the *Salmonella* mutagenicity test. The active mutagenic substances were aromatic diamines and aminophenols, and most of these needed metabolism by mammalian enzymes to be converted to the mutagenic

Abbreviations: 2,4-DAA = 2,4-diaminoanisole; 2,4-DAT = 2,4-diaminotoluene; BNF = β-naphthoflavone; 2-FA = 2-fluorenamine; 2-FAA = 2-fluorenylacetamide; AHH = aryl hydrocarbon hydroxylase; 3-MCA = 3-methylcholanthrene.

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forms (*11*). Considerable differences with respect to in vitro mutagenic activity were noted among the various substances, but 2,4-DAA was the most potent of the diamines. Those diamines containing a nitro group were also directly mutagenic, due to the additional activation by bacterial enzymes (*11*).

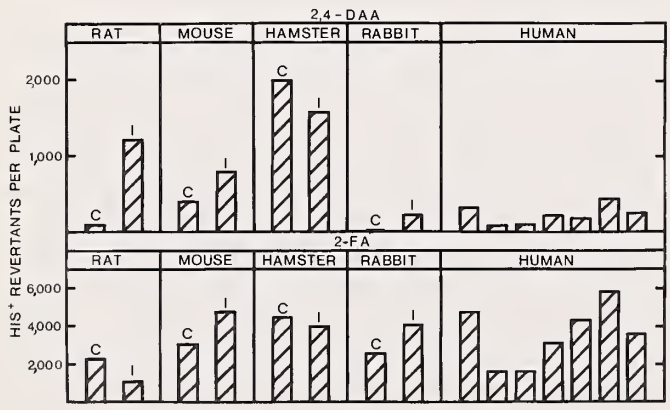
Long-term feeding studies with rats and mice were performed by investigators at the National Cancer Institute to determine whether these mutagenic hair dye ingredients also have the potential for causing cancer in animals. Of the compounds tested, 2,4-DAA sulfate, 2,4-DAT, and 1,2-diaminobenzene were carcinogenic in both species, whereas 2-nitro-1,4-diaminobenzene was carcinogenic in mice and 2-nitro-4-aminophenol was carcinogenic in rats (*6, 7*). Other compounds, e.g., 4-nitro-1,2-diaminobenzene, 1,3-diaminobenzene, 1,4-diaminobenzene, and 2,5-DAT, which have been shown to be mutagenic, were carcinogenic (*6, 7, 11, 12*). When it was established that 2,4-DAT caused liver carcinomas in long-term feeding studies in rats (*8*), it was withdrawn from use in hair dyes sold on the United States market.

The liver microsomal enzyme activities involved in converting 2,4-DAA to a bacterial mutagen can be increased by pretreatment of the animals with inducers of the polycyclic aromatic hydrocarbon class. Both basal levels of activity and the relative inducibility show considerable species differences when liver fractions from several laboratory animals are used (text-fig. 1). Mutagenic activation of 2,4-DAA was also studied with the use of subfractions prepared from the livers of 7 humans who served as kidney transplant donors (text-fig. 1). With the preparations from laboratory animals, the highest basal activites of 2,4-DAA mutagenicity were seen with hamster liver, whereas rabbits had the lowest.

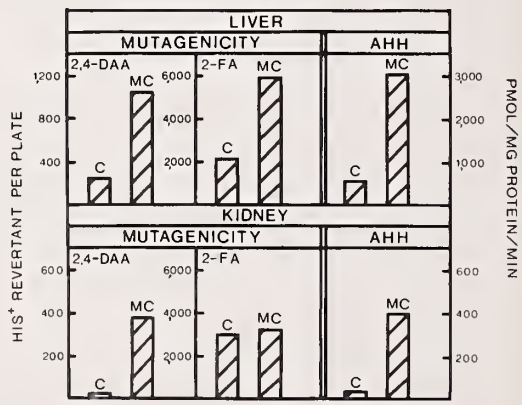
Pretreatments with BNF were most effective in rats; under the experimental conditions used, BNF treatment led to a decrease in mutagenic activity with hamster liver subfractions. These findings, when compared with the model hepatocarcinogen 2-FA, revealed that basal activities with 2-FA were much higher than with 2,4-DAA; again, hamster liver was the most active preparation. Pretreatment with BNF led to an increase with mouse and rabbit liver subfractions, whereas a decrease in activity was noted with fractions from rats and hamsters. The effect of inducer treatment on mutagenic activation is presumably the net result of the induction of both activating and detoxifying pathways, if we assume that mutagen concentrations are not saturating. Considerable interindividual differences occurred in the activities of the human liver preparations with 2,4-DAA and 2-FA as substrates; the highest activities were approximately five times those of the lowest. The levels of activity demonstrated by the human livers were about the same as those of the common laboratory animals. Furthermore, the activity of 2,4-DAA and 2-FA correlated, which suggested similar rate-limiting activation pathways.

Human liver microsomes have metabolized 2-FAA by both detoxification (ring hydroxylation) and activation (N-hydroxylation) pathways (*13, 14*). For these reactions also, considerable interindividual differences in activity were noted (*14*). The relative degree of in vitro mutagenic activation of 2-FAA and that of 2,4-DAA and 2-FA among the liver samples was similar to the variations in 2-FAA N-hydroxylase activities, a finding that suggested that N-hydroxylation may be the rate-limiting step in the activation process for the 3 compounds (*14*).

Studies by Nebert and co-workers (*15, 16*) have shown that association between induction of cytochrome P_{448} and a multitide of mono-oxygenase activities such as AHH in mice treated with polycyclic aromatic hydrocarbons is genetically linked. When liver fractions from 3-MCA-pretreated, aromatic hydrocarbon-responsive mice were used, it was found that 2,4-DAA activation to a mutagen was increased 18 times over that detected when uninduced fractions were used, whereas 2-FA mutagenicity was increased 5 times (text-fig. 2). These effects were associated with a fivefold increase in liver microsomal AHH activity. A large increase in 2,4-DAA mutagenicity was seen when kidney fractions from mice treated with 3-MCA



TEXT-FIGURE 1.—Ten micrograms of 2,4-DAA or 2-FA was plated with 2 mg control or BNF-induced liver 9,000×*g* supernatant protein with *Salmonella typhimurium* TA98. *HIS*⁺ = histidine prototrophy.



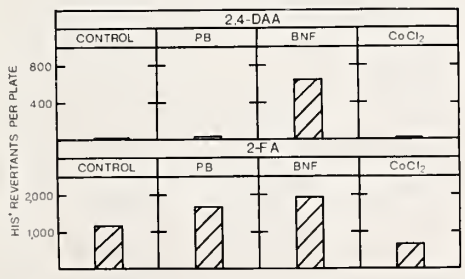
TEXT-FIGURE 2.—Ten micrograms of 2,4-DAA or 2-FA was plated with 2 mg control or 3-MCA-induced liver 9,000×*g* supernatant protein or 1 mg control or 3-MCA-induced kidney 9,000×*g* supernatant protein from C57BL/6 mice with *S. typhimurium* TA1538. AHH activity was measured with 80 μM benzo[*a*]pyrene and 0.2 mg/ml microsomal protein.

were compared with those from the uninduced animals; this correlated well with a tenfold increase in kidney microsomal AHH activity after treatment with 3-MCA. Mutagenic activation of 2-FA with kidney fractions gave a different picture; basal activity was high, higher, in fact, than with liver fractions, but no significant increase was seen after the treatment with the inducing agent. This finding was also evident in separate experiments when both protein and mutagen concentrations were varied. Thus the difference in the pattern of metabolic activation of these two arylamines with respect to constitutive activities and to the response to aromatic hydrocarbons (*17*) is clear.

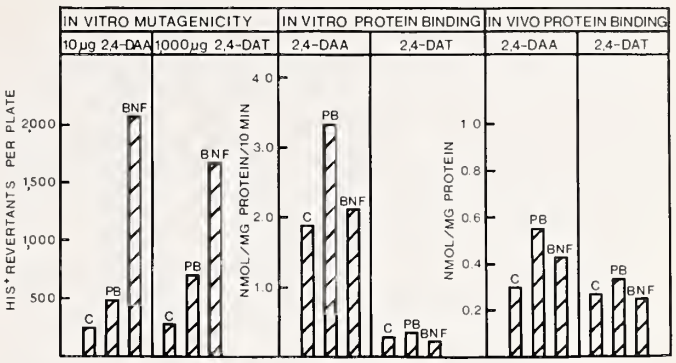
Rat liver microsomes usually contain the highest specific activities of mono-oxygenases associated with cytochrome P_{450} when compared with other subfractions and organs. Results of several recent studies revealed that isolated rat liver nuclei also possess cytochrome P_{450} and that this enzyme system participates in reactions such as the covalent binding of benzo[*a*]pyrene to nuclear DNA (*18–20*). Formation of reactive intermediates by cytochrome P_{450} enzymes in the nuclear membrane might be important because of the short distance such intermediates would have to traverse to reach nuclear DNA.

The system of isolated rat liver nuclei has been used in the study of the mutagenic activation of 2,4-DAA and 2-FA (text-fig. 3). Nuclei from untreated rat liver had minimal potency in activating 2,4-DAA to a mutagen. However, with nuclei from BNF-treated rats, a marked increase in mutagenicity was seen. In contrast, considerable activity was noted with 2-FA with control nuclei, and a moderate rise resulted after pretreatment with phenobarbital and BNF. Treatment with cobaltous chloride, a cytochrome P_{450} inhibitor, led to a decrease in 2-FA mutagenic activity. The mutagenic activation of the two arylamines, both with respect to basal activities and to the response to inducers, was markedly different in this system.

Several studies have shown that considerable differences among the various diamines contained in hair dyes occur in their in vitro mutagenic potency (*11, 12*). Text-figure 4 demonstrates that 2,4-DAA is approximately 100 times more potent than 2,4-DAT in this respect, and it was also evident that BNF was a much better inducer of mutagenic activation of the diamines than was phenobarbital. As another measure of formation of reactive intermediates after metabolism of the diamines, covalent binding to protein has been determined (*21–23*). The in vitro covalent binding rate of 2,4-DAA to microsomal protein was seven times higher compared with that of 2,4-DAT (text-fig. 4). In contrast to the effects on mutagenic activation, pretreatment with BNF did not increase covalent binding rates, whereas that of phenobarbital did. When the radiolabeled diamines were given to animals, 2,4-DAA and 2,4-DAT were converted to products which bound covalently to liver protein; and the levels of binding were also similar for the 2 compounds (text-fig. 4). These results show that the correlation between in vitro and in vivo potency in metabolic activation reactions is not always good. The differences in effects of inducers on mutagenicity compared with covalent binding of the diamines indicate that the pathways involved in mutagenicity and covalent binding vary.



TEXT-FIGURE 3.—The 0.05 mM of 2,4-DAA or 2-FA was preincubated with 2 mg/ml control or pretreated rat liver nuclear protein with *S. typhimurium* TA1538 for 30 min and then plated. $CoCl_2$ = cobaltous chloride; PB = phenobarbital.



TEXT-FIGURE 4.—Mutagenicity was determined with *S. typhimurium* TA1538 and 2 mg control or pretreated rat liver 9,000×*g* supernatant protein. In vitro protein binding was determined with 0.5 mM of [^{3}H]2,4-DAA or [^{3}H]2,4-DAT and 2 mg control or pretreated rat liver microsomal protein/ml. In vivo protein binding in rat liver was determined 4 hr after ip administration of 100 mg [^{3}H]2,4-DAA or [^{3}H]2,4-DAT/kg to control (C) or pretreated animals. PB = phenobarbital.

STUDIES WITH PHENACETIN

The acetanilide analgesic and antipyretic phenacetin has been widely used in medicinal mixtures for the last 80 years. Consumption has been considerable and, largely through self-medication, there has been widespread abuse of this drug. Increased frequency and duration of exposure has in some instances led to a total lifetime consumption of 29 kg of phenacetin (*24*).

Several types of human renal disease have been associated with prolonged use of this acetanilide drug. In 1953, Spühler and Zollinger (*25*) first reported that phenacetin was implicated in the production of papillary necrosis of the kidney. After epidemiologic studies in Sweden in 1965, it became evident that ingestion of phenacetin-containing analgesic mixtures may lead to the formation of transitional cell carcinomas of the renal pelvis and lower urinary tract (*26*). In a report of 62 patients with carcinoma of the renal pelvis who were known phenacetin abusers, the average total dose was 9.1 kg and average exposure and induction times were 17 and 22 years, respectively (*27*). In 59 of these patients, papillary necrosis was a prominent feature;

8 patients were also reported to have tumors of the urinary bladder.

Although an earlier long-term study of rats given phenacetin did not reveal a carcinogenic effect (*28*), administration to rats of 1.25 and 2.5% phenacetin in the diet recently resulted in the formation of nasal cavity carcinomas (*29*). Also, feeding of *N*-hydroxyphenacetin (a potential metabolite) resulted in the development of a high incidence of hepatocellular carcinomas in rats (*30*).

Phenacetin is metabolized through various oxidative, hydrolytic, and conjugative pathways (*24*). However, the major metabolic route is through *O*-dealkylation, whereby acetaminophen, the active analgesic metabolite of phenacetin, is formed. Acetaminophen is further conjugated with glucuronic and sulfuric acids. A minor route is through hydrolysis of the acetamido bond which results in the formation of *p*-phenetidine. Both *p*-phenetidine and phenacetin can undergo ring hydroxylation at the 2-position, and the corresponding products can subsequently form sulfate esters. Phenacetin, acetaminophen, and *p*-phenetidine have all been considered substrates in N-hydroxylation reactions.

Several metabolic pathways leading to the formation of reactive intermediates from phenacetin can be envisioned. Some of these intermediates can combine with glutathione and form the mercapturic acid conjugate of acetaminophen. One activation pathway could be the N-hydroxylation of acetaminophen; a second, conjugation of *N*-hydroxyphenacetin with glucuronate or sulfate; and a third possibility is the formation of phenacetin 3,4-epoxide. Hinson et al. (*31*) established that de-ethylation of phenacetin to acetaminophen, followed by activation of acetaminophen, is the predominant pathway in the formation of acetaminophen mercapturate in hamsters.

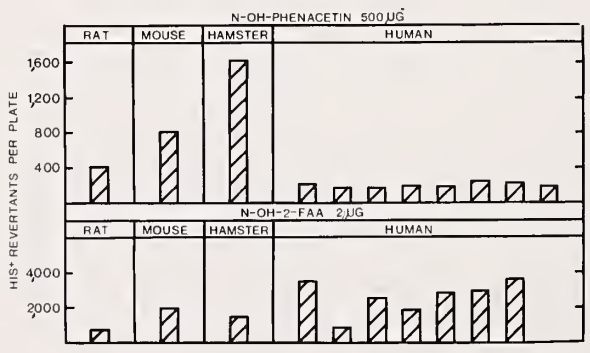
Studies by Thorgeirsson and collaborators (*32, 33*) have established a role for deacetylation in the microsomal activation of the hydroxamic acids, *N*-hydroxy-2-acetylaminofluorene and *N*-hydroxyphenacetin, to mutagens in vitro. Species variations were considerable with respect to activation of *N*-hydroxyphenacetin to a mutagen by liver microsomes (text-fig. 5). Among laboratory animals, hamsters had the highest activity, mice were intermediate in their response, and rats had the lowest activity. Human liver microsomes also converted *N*-hydroxyphenacetin to a mutagen with activities slightly less than that of rat liver microsomes. A different pattern was noted for the liver microsomal activation of *N*-hydroxy-2-FAA to a mutagen. Preparations from mice were more active than those from hamsters and considerably more so than those from rats. Six of the 7 human liver samples had higher activities than any of the laboratory animals.

Use of cell transformation systems as a short-term screen in the prediction of carcinogenicity of environmental chemicals is an encouraging approach. Furthermore, such systems are helpful in the study of mechanisms of chemical carcinogenesis. Studies by Heidelberger and associates (*34, 35*) with C3H/10T1/2 clone 8 mouse embryo fibroblasts have shown that these cells are transformed by compounds such as benzo[*a*]pyrene, 3-MCA, and 7,12-dimethylbenz[*a*]anthracene. The transformed cells developed into fibrosarcomas after injection into syngeneic mice.

The C3H/10T1/2 cell transformation system was used in the determination of the transforming potential of *N*-hydroxyphenacetin. In cytotoxicity assays, the cells were seeded at 60 cells/ml in 5-ml cultures. Twenty-four hours after seeding, fresh medium containing *N*-hydroxyphenacetin at several concentrations was added, and the cells were exposed for 24 hours before fresh medium was added. Seven days after seeding, the cultures were fixed and stained with Giemsa, and the number of foci per plate were counted. At each concentration, 6 cultures were exposed.

The transformation system and the scoring of the three types of transformed foci were as reported by Reznikoff et al. (*35*). *N*-Hydroxyphenacetin was dissolved in acetone, but the control series contained solvent only. The C3H/10T1/2 cells were seeded at 500 cells/ml in 5-ml cultures, and the foci were counted after 6 weeks; at each concentration 12 cultures were exposed.

The cytotoxic effect of *N*-hydroxyphenacetin increased slowly as the concentration increased, and 50% inhibition of colony formation occurred at approximately 75 μg/ml (table 1). The transformation of the C3H/10T1/2 cells was mainly into the nononcogenic type I cells, but type III cells, which may be oncogenic, were also clearly evident. An investigation of the presence of short and long microvilli of these cells is presently being undertaken. Previ-



TEXT-FIGURE 5.—Mutagenicity was determined with *S. typhimurium* TA100 and 0.7 mg *N*-hydroxyphenacetin or 0.4 mg *N*-hydroxy-2-FAA liver microsomal protein.

TABLE 1.—*Cytotoxic effects and transforming activity of N-hydroxyphenacetin in C3H/10T1/2 cells*

Series	*N*-Hydroxyphenacetin, μg/ml	Cytotoxicity, colonies/plate[a]	Transforming activity: No. of transformed foci/plate	Transforming activity: Type III foci/plate
1	0	78	0	0
2	0.1	71 (91)	3.1	0.1
3	1.0	65 (83)	4.6	0.6
4	10	57 (73)	6.5	1.1
5	50		4.4	1.6
6	100	36 (46)		
7	500	5 (6)		

[a] *Numbers in parentheses* are percentages of Series 1.

ously, the occurrence of long microvilli was described as a marker of the oncogenic potential of transformed cells (*36, 37*).

REFERENCES

(*1*) Bengtsson U, Angervall L, Akman H, et al: Transitional cell tumors of the renal pelvis in analgesic abusers. Scand J Urol Nephrol 2:145–150, 1968

(*2*) Angervall L, Bengtsson U, Zetterland CG, et al: Renal pelvic carcinoma in a Swedish district with abuse of a phenacetin-containing drug. Br J Urol 41:401–405, 1969

(*3*) Kinlen L, Harris R, Garrod A, et al: Use of hair dyes by patients with breast cancer: A case-control study. Br Med J 1:366–368, 1977

(*4*) Neutel C, Nair R, Last J: Are hair dyes associated with bladder cancer? Can Med Assoc J 119:307–308, 1978

(*5*) Shore RE, Pasternack BS, Thiessen EU, et al: A case-control study of hair dye use and breast cancer. J Natl Cancer Inst 62:277–283, 1979

(*6*) Carcinogenesis Testing Program, National Cancer Institute: Carcinogenesis Tech Rep No. 84, 94, 126; 162, 169, 174, 180. Washington, D.C.: U.S. Govt Print Off, 1978, 1979

(*7*) Weisburger EK, Russfield AB, Homburger F, et al: Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. J Environ Pathol Toxicol 2:325–356, 1978

(*8*) Ito N, Hiasa Y, Konishi Y, et al: The development of carcinoma in livers of rats treated with *m*-toluylenediamine and the synergistic and antagonistic effects with other chemicals. Cancer Res 29:1137–1145, 1969

(*9*) Hanlon J: Tint of suspicion. New Scientist 78:352–356, 1978

(*10*) Corbett J: The role of *meta* difunctional benzenes in oxidative hair dyeing. Part I. Reaction with *p*-diamines. J Soc Cosmetic Chemists 24:103–134, 1973

(*11*) Ames BN, Kammen HO, Yamasaki E: Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. Proc Natl Acad Sci USA 72:2423–2427, 1975

(*12*) Dybing E, Thorgeirsson SS: Metabolic activation of 2,4-DAA, a hair-dye component. I. Role of cytochrome P_{450} metabolism in mutagenicity in vitro. Biochem Pharmacol 26:729–734, 1977

(*13*) Weisburger JH, Grantham PH, Vanhorn F, et al: Activation and detoxification of *N*-2-fluorenylacetamide in man. Cancer Res 24:475–479, 1964

(*14*) Dybing E, von Bahr C, Aune T, et al: In vitro metabolism and activation of carcinogenic aromatic amines by subcellular fractions of human liver. Cancer Res 29:4206–4211, 1979

(*15*) Nebert DW, Robinson JR, Niwa A, et al: Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse. J Cell Physiol 85:393–414, 1975

(*16*) Thorgeirsson SS, Nebert DW: The *AH* locus and the metabolism of chemical carcinogens and other foreign compounds. Adv Cancer Res 25:149–193, 1977

(*17*) Aune T, Dybing E: Mutagenic activation of 2,4-diaminoanisole and 2-aminofluorene in vitro by liver and kidney fractions from aromatic hydrocarbon responsive and nonresponsive mice. Biochem Pharmacol 28:2791–2797, 1979

(*18*) Rogan E, Cavalieri E: Differences between nuclear and microsomal cytochrome P_{450} in uninduced and induced rat liver. Mol Pharmacol 14:215–219, 1978

(*19*) Rogan EG, Mailander P, Cavalieri E: Metabolic activation of aromatic hydrocarbons in purified rat liver nuclei: Induction of enzyme activities and binding to DNA with and without mono-oxygenase-catalyzed formation of active oxygen. Proc Natl Acad Sci USA 73:457–461, 1976

(*20*) Jernström B, Badi H, Orrenius S: Formation in isolated rat liver microsomes and nuclei of benzo[*a*]pyrene metabolites that bind to DNA. Cancer Res 36:4107–4113, 1976

(*21*) Dybing E, Aune T, Nelson SD: Metabolic activation of 2,4-diaminoanisole, a hair-dye component. II. Role of cytochrome P_{450} metabolism in irreversible binding in vitro. Biochem Pharmacol 28:43–50, 1979

(*22*) _____: Metabolic activation of 2,4-diaminoanisole, a hair-dye component. III. Role of cytochrome P_{450} metabolism in irreversible binding in vivo. Biochem Pharmacol 28:51–55, 1979

(*23*) Aune, T, Nelson SD, Dybing E: Mutagenicity and irreversible binding of the hepatocarcinogen, 2,4-diaminotoluene. Chem Biol Interact 25:23–33, 1979

(*24*) Carro-Ciampi G: Phenacetin abuse: A review. Toxicology 10:311–339, 1978

(*25*) Spühler O, Zollinger HU: Die chronisch-interstitielle Nephritis. Z Klin Med 151:1–50, 1953

(*26*) Hultengren N, Lagergren C, Ljungqvist A: Carcinoma of the renal pelvis in renal papillary necrosis. Acta Chir Scand 130:314–320, 1965

(*27*) Bengtsson U, Angervall L, Johansson S, et al: Phenacetin abuse and renal pelvic carcinoma. Int J Clin Pharmacol 12:290–294, 1975

(*28*) Schmahl D, Reiter A: Fehlen einer cancerogenen Wirkung beim Phenacetin. Arzneim Forsch 4:404–405, 1954

(*29*) Isaka H, Yoshii H, Otsuji A, et al: Tumors of Sprague-Dawley rats induced by long-term feeding of phenacetin. Gan 70:29–36, 1979

(*30*) Calder IC, Goss DE, Williams PJ, et al: Neoplasia in the rat induced by *N*-hydroxyphenacetin, a metabolite of phenacetin. Pathology 8:1–6, 1976

(*31*) Hinson JA, Nelson SD, Gillette JR: Metabolism of (p-^{18}O)-phenacetin: The mechanism of activation of phenacetin to reactive metabolites in hamsters. Mol Pharmacol 15:419–427, 1979

(*32*) Schut HA, Wirth PJ, Thorgeirsson SS: Mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system: The role of deacetylation by liver and kidney fractions from mouse and rat. Mol Pharmacol 14:682–692, 1978

(*33*) Wirth PJ, Dybing E, von Bahr C, et al: Mechanism of N-hydroxy-acetylarylamine mutagenicity in the *Salmonella* test system: Metabolic activation of *N*-hydroxyphenacetin by liver and kidney fractions from the rat, mouse, hamster, and man. Mol Pharmacol 18:117–127, 1980

(*34*) Reznikoff CA, Brankow DW, Heidelberger C: Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res 33:3231–3238, 1973

(*35*) Reznikoff CA, Bertram JS, Brankow DW, et al: Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res 33:3239–3249, 1973

(*36*) Saxholm HJ: The oncogenic potential of three different 7,12-dimethylbenz[*a*]anthracene transformed C3H/10T/1/2 cell clones at various passages and the impor-

tance of the mode of immunosuppression. Eur J Cancer 15:515–526, 1979

(*37*) SAXHOLM HJ, REITH A: The surface structure of 7,12-dimethylbenz[*a*]anthracene transformed C3H/10T/1/2 cells. A quantitative scanning electron microscopical study. Eur J Cancer 15:843–855, 1979

Carcinogenic, Mutagenic, and Comutagenic Aromatic Amines in Human Foods[1]

Takashi Sugimura and Minako Nagao[2]

ABSTRACT—Three recent topics related to possible exposure of humans to mutagenic and carcinogenic aromatic amines and related compounds in foods are reviewed. A food additive, AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, was first demonstrated to be mutagenic in *Escherichia coli* WP-2 and then proved to be carcinogenic in experimental animals. This is an example of prediction of the carcinogenicity of a compound from results of short-term microbial tests. Pyrolysates of amino acids, proteins, and foods high in a protein contain many heterocyclic aromatic amine compounds: For example, a tryptophan pyrolysate contains two derivatives of amino-γ-carboline (pyridoindole), and a glutamic acid pyrolysate contains two derivatives of dipyridoimidazole. These compounds are strong frameshift mutagens in *Salmonella typhimurium*. Some of them were carcinogenic in an in vitro transformation test and were also carcinogenic when injected sc into hamsters and rats and when given orally to mice. Carcinogenic aromatic amines, such as aniline, and *o*-toluidine and yellow OB were demonstrated to be mutagenic in the presence of the β-carboline, norharman, with S-9 mix. Diphenylnitrosamine was also mutagenic in the presence of norharman, which is present in tobacco tar and broiled food. These mutagenicities of aniline, *o*-toluidine, yellow OB, and diphenylnitrosamine are discussed in relation to an evaluation of compounds as environmental carcinogens from the results of short-term microbial tests.—Natl Cancer Inst Monogr 58: 27–33, 1981.

Aromatic amines are present in many parts of the human environment, including food. We have reviewed the work on the food additive AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide. This heterocyclic nitrocompound is not an aromatic amine in the strict sense, but it possesses many features in common with aromatic amines; AF-2 is a typical example of a compound that was first shown to be mutagenic in microbial tests and later confirmed to be carcinogenic in animal tests in vivo. Its qualities raise a question of current interest; i.e., what should be done about controlling a compound when its mutagenicity has been confirmed but its carcinogenicity has not yet been demonstrated?

This article also covers the characteristics of mutagens and carcinogens in pyrolysates of amino acids, proteins, and foods rich in proteins. A few years ago we (*1, 2*) reported that the charred parts of fish and meat have mutagenic activity which cannot be fully accounted for by the content of benzo[*a*]pyrene. Later, many new chemicals were found in pyrolysates of amino acids (*3–6*) and proteins (*7*), and some of them were present in the charred parts of fish and meat (*8*); most are derivatives of heterocyclic aromatic amines. The specific mutagenic activities of Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 were similar to or more than that of aflatoxin B_1 toward *Salmonella typhimurium* TA98 with metabolic activation (*3, 5, 9*). These four strong mutagens have been synthesized chemically (*10, 11*), and some of them were demonstrated carcinogens in vitro and in vivo (*12–14*). Recently, the activations and inactivations of Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, 2-amino-3-methyl-α-carboline, and 2-amino-α-carboline have been studied (*15–17*). Inasmuch as this field of research is developing so quickly, a brief review of these studies is given here.

The third topic to be discussed is the comutagenic action of the β-carboline, nonharman, on compounds related to aromatic amines (*18, 19*). Some carcinogenic aromatic amines are not mutagenic alone but are mutagenic in the presence of norharman. Norharman is 1) nonmutagenic, 2) found in tobacco tar (*20*) and the charred part of protein foods (*21*), and 3) produced in high yield by pyrolysis of tryptophan (*21*). Diphenylnitrosamine, shown to induce carcinomas in experimental animals in vivo (*22*), was also demonstrated to be mutagenic in the presence of norharman (*23*).

MATERIALS AND METHODS

Chemicals.—Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and amino-α-carboline were purchased from the Nard Chemical Co., Osaka. The sources of other chemicals used are given in the respective references cited in this article (*24–41*).

Microbial test.—*S. typhimurium* TA98 and TA100 were used primarily (*42*). TA98 is a frameshift mutant and TA100 is a base-pair exchange mutant (*42*); both organisms possess plasmid pKM101. Microbial tests were done by a preincubation method, which is a modification of Ames' procedure. The bacterial suspension, S-9 mix, and

Abbreviations: AF-2 = 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; Trp-P-1 = (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole); Trp-P-2 = (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole); Glu-P-1 = (2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2 = (2-aminodipyrido[1,2-α:3′,2′-*d*]imidazole); Lys-P-1 = 3,4-cyclopentenopyrido-[3,2-α]carbazole; Phe-P-1 = 2-amino-5-phenylpyridine.

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the test substance were preincubated for 20 minutes at 37° C (*43*). Then molten soft agar was added, and the mixture was poured onto an agar plate.

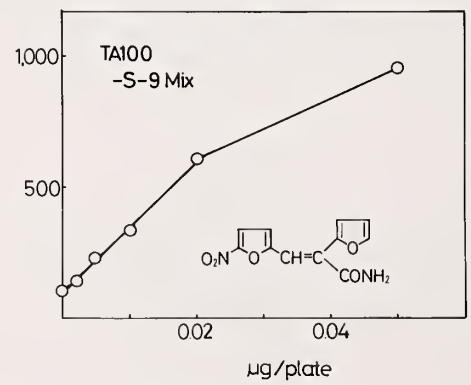
Animal experiments.—The species and strains of animals used and the conditions of administration of mutagens are described in detail in the original papers cited (*14, 32–37, 44–46*).

RESULTS AND DISCUSSION

The Food Additive AF-2: A Mutagenic Carcinogen

Text-figure 1 shows that the specific mutagenic activity of AF-2 in TA100 is extremely high. The mutagenicity of AF-2 was first demonstrated in *Escherichia coli* (*24, 25*), and later AF-2 was shown to give positive results in *rec*-assay with the use of *Bacillus subtilis* strain *rec*$^+$ and *rec*$^-$ (*25*) and *Saccharomyces cervisiae* (*26*). In mammalian cell cultures, AF-2 produced chromosomal aberrations (*27*) and mutations (*28*). However, it is interesting to recall that the mutagenicity of AF-2 was not observed with *S. typhimurium* TA1535, TA1536, TA1537, or TA1538 (*29*). TA100 and TA98, obtained by the introduction of plasmids into TA1535 and TA1538, respectively, were sensitive to the mutagenic activity of AF-2 (*30*). The fact that incorporation of plasmids made *Salmonella* sensitive to AF-2 may be due to the enhancement of the Weigle reactivation by pKM101 (*31*).

When positive data on the mutagenicity of AF-2 and negative data on its carcinogenicity were available, the decision on whether to ban the usage of AF-2 as a food additive was difficult for the Ministry of Health from a regulatory viewpoint. Most biochemists and geneticists were in favor of banning its use: The biochemists suspected that mutagenic AF-2 would later prove to be carcinogenic, whereas the geneticists were worried that AF-2 might produce germinal cell mutation in humans. On the other hand, most pathologists were reluctant to ban the usage of AF-2, mainly because a pathologist's results on in vivo carcinogenesis tests in rats fed an AF-2 diet failed to demonstrate its carcinogenicity (*32*). However, in the midst of the heated discussion among scientists, consumers, government officials, industrial people, and journalists, other investigators (*33*) reported that oral AF-2 was indeed carcinogenic to mice, and the incidences of squamous cell carcinomas of the forestomach of mice fed diets containing 0.15 and 0.45% AF-2 were 10 and 30%, respectively. On the basis of this new evidence of its carcinogenicity, AF-2 was immediately banned. Afterward, the carcinogenicity of AF-2 was demonstrated in hamsters, mice, and rats (*34–37*). Even now, there is still no evidence that AF-2 affects the germinal cells of animals: In other words, the demonstration that a certain chemical was mutagenic in microbes and caused chromosome aberrations in cultured cells was useful for the prediction of its carcinogenicity in vivo but not its mutagenicity in germ cells. Therefore, demonstration of the mutagenicity of food is now more directly concerned with potential carcinogenicity than with a potential genetic hazard to the next generation.



TEXT-FIGURE 1.—Mutagenicity of AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide. Ordinate = No. of *his*$^+$ revertants.

The magnitudes of the specific mutagenicities of chemicals vary widely in a 7-power range on a logarithmic scale (*47*); AF-2 has one of the highest known specific mutagenicities. Now everybody unhesitatingly predicts the carcinogenicity of AF-2 from data on its mutagenicity. However, a concentration of 5–25 ppm of AF-2 was permissible in foods in Japan, and a simple calculation indicates that the average intake per capita was 0.72 mg/kg. According to results of experiments on the hamster, this average intake by humans is estimated as about 0.0005 of the TD50 value (*38*), as shown in table 1. Thus although AF-2 is a typical mutagen in our food that was later proved to be carcinogenic, its real impact as a human hazard is still unclear, and no epidemiologic data are available to clarify this point.

Consumers became aware of the problem of food additives after attention was drawn to AF-2. If any food additives are mutagenic, consumers in Japan now believe that they will cause human cancer. However, consumers do not usually pay much attention to the quantity of these additives in our food or to their specific mutagenicity. Scientists must and do think differently from consumers; they must struggle with the difficult quantitative estimation of human risks caused by mutagens and carcinogens in foods. The gap between consumers and scientists seems to have become wider.

TABLE 1.—*Estimation of the carcinogenic risk to humans by AF-2*

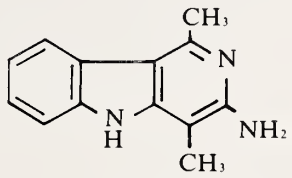
Animal	AF-2 intake TD50, mg/kg/day, animals	Estimated risk, B:A,[a] humans
Hamster	31	1:2,600
Rat	24	1:2,000
Mouse	170	1:14,000

[a] A is TD50 in milligrams/kilogram/day. B is 0.012 mg/kg/day and is the average intake of AF-2 by a Japanese in 1973. This value was obtained from the total annual production of AF-2, which, in 1973, was 2.7 tons.

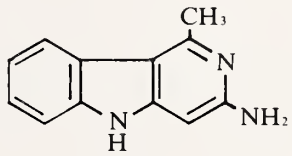
Mutagens in Foods Produced by Cooking

The charred parts of broiled fish and meat were mutagenic to *S. typhimurium* (*1*, *2*, *48–51*), but the actual content of aromatic hydrocarbons could not account for their mutagenic potentials. The mutagenicity was found mostly in the basic fraction of tars. Two crystalline substances were isolated from a pyrolysate of DL-tryptophan, and their structures were determined. They were Trp-P-1 and Trp-P-2 (*3*, *4*). Also obtained from a glutamic acid pyrolysate were Glu-P-1 and Glu-P-2 (*5*). In addition, Lys-P-1 was identified in a lysine pyrolysate (*6*), and Phe-P-1 was isolated from a phenylalanine pyrolysate (*3*, *4*); 2-amino-α-carboline and 2-amino-3-methyl-α-carboline were obtained from a pyrolysate of soybean globulin (*7*), and benzo[*f*]quinoline and phenanthridine were isolated from a pyrolysate of proteins (*4*). These structures are given in text-figure 2.

All these compounds were mutagenic with metabolic activation by the S-9 fraction. The specific mutagenicities of



Trp-P-1



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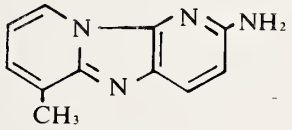
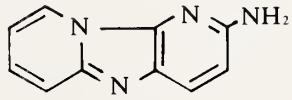
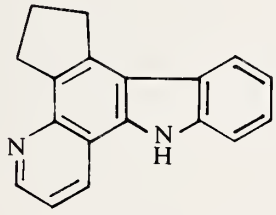


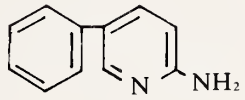
Glu-P-1



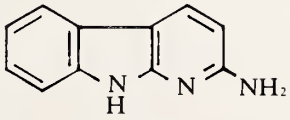
Glu-P-2



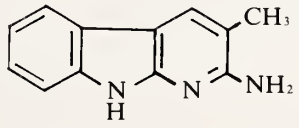
Lys-P-1



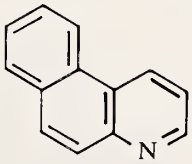
Phe-P-1



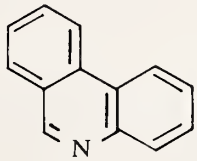
2-Amino-α-carboline



2-Amino-3-methyl-α-carboline



Benzo[*f*]quinoline



Phenanthridine

TEXT-FIGURE 2.—Structures of mutagens in pyrolysates of amino acids and proteins.

TABLE 2.—*Specific mutagenic activity of mutagens in pyrolysates of amino acids and proteins*

Compound	Amount of S-9/plate, μl	No. of revertants of *his*⁺/μg	
		TA98	TA100
Trp-P-2	10	104,000	1,800
Glu-P-1	30	49,000	3,200
Trp-P-1	10	39,000	1,700
Glu-P-2	30	1,900	1,200
2-Amino-α-carboline	150	300	20
2-Amino-3-methyl-α-carboline	"	200	120
Lys-P-1	"	86	99
Phe-P-1	"	41	21
Benzo[*f*]quinoline	"	0.69	7.4
Phenanthridine	"	0.57	2.9

these compounds are given in table 2. We found that the specific mutagenic activities of Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 were in the same order of magnitude as that of aflatoxin B_1 toward *S. typhimurium* TA98 and were similar to those of 4-nitro-quinoline 1-oxide and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine toward TA100 (*9*). The specific mutagenic activity of Lys-P-1 was between those of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene toward *S. typhimurium* TA98 and TA100 (*6*). Other mutagenic compounds are also in the basic fraction of cooked fish and meats, and their structures have been proposed to be imidazoquinoline derivatives (*52*).

Also found in pyrolysates of casein and albumin were Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 (*21, 53, 54*). The actual contents of these compounds in broiled fish and meat that are now being measured quantitatively by partial purification followed by gas–mass spectrometry (*8*) probably vary with the conditions of broiling and with the part of the meat or fish charred. However, one recent experiment indicated that fish that had been sun dried and broiled in the normal Japanese way contained about 13 ng/g each of Trp-P-1 and Trp-P-2 (*8*).

Mutagenic also to cultured mammalian cells, Trp-P-1 and Trp-P-2 produced sister chromatid exchanges in cultured human lymphoblastoid cells and caused transformation in vitro of cultured Syrian hamster embryo cells (*12, 13, 55*). Adducts of Trp-P-2 and Glu-P-1 with guanine bases were demonstrated (*56, 57*). When injected sc into rats and hamsters (*14*) Trp-P-1 induced fibrosarcomas, and pellet diets containing Trp-P-1 or Trp-P-2 induced hepatomas in mice (*44*). Cytochrome P_{450} activated Trp-P-1 and Trp-P-2 (*58, 59*).

The carcinogenic activities of Trp-P-1 and Trp-P-2 are weaker than expected from their strong mutagenicities, but these compounds are definitely carcinogenic when administered locally or systemically.

Extracts of some vegetables inactivated Trp-P-1 and Trp-P-2, probably by the actions of hemoproteins (*15*); myeloperoxidase and hydrogen peroxide reacted similarly (*16*). Under acidic conditions, like those in the stomach, these compounds were converted to deaminated compounds by a low concentration of nitrite (*60*). In addition, the mutagenicities of Trp-P-1 and Trp-P-2 were enhanced by cysteamine or cysteine ethylester (*17*) and reduced by hemin (*61*).

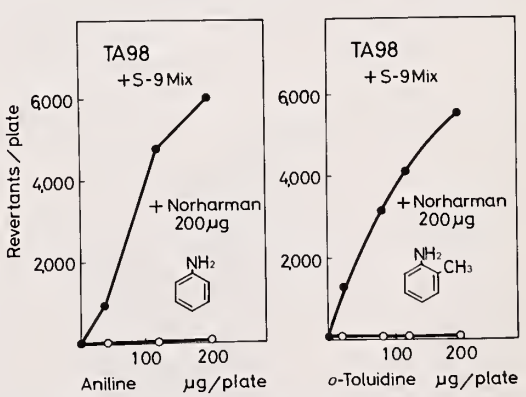
The actual concentrations of Trp-P-1 and Trp-P-2 seem to be low in prepared foods, but more precise analyses of their exact amounts are required. We should also note that only a small portion of the total mutagenicity of broiled fish or meat is accounted for by Trp-P-1 and Trp-P-2, and thus quantitative and qualitative studies are required on other mutagens in these foods. Because these heterocyclic aromatic amines with mutagenicity and carcinogenicity are actually produced during cooking, consideration should be given to their actual importance as a cause of human cancer.

Comutagenicity of Aromatic Amines

During purification of Trp-P-1 and Trp-P-2 from a tryptophan pyrolysate, the mutagenic activity decreased greatly at the step of separation of norharman and harman from the fraction containing Trp-P-1 and Trp-P-2. Reconstitution of the fraction containing norharman with that containing Trp-P-1 or Trp-P-2 restored the original mutagenic activity (*39*). This was especially true when a large amount of S-9 was used (*62*). Considerable norharman is produced by pyrolysis of tryptophan (*4*). Norharman has no mutagenic activity toward *S. typhimurium* TA100 or TA98 even in the presence of S-9 mix. The striking effects of norharman on aniline and *o*-toluidine are shown in text-figure 3; the latter compounds were not mutagenic by the Ames test under ordinary experimental conditions but became mutagenic when preincubated with norharman and S-9 mix. Dose-dependent mutagenicities of aniline and *o*-toluidine were observed with norharman and S-9.

Aniline has been a suspected carcinogen to humans from epidemiologic data, and results of experiments demonstrated the carcinogenicity of oral aniline (*45*) and *o*-toluidine (*46*) in rats.

Inasmuch as norharman was not mutagenic and carcinogens such as aniline and *o*-toluidine were mutagenic



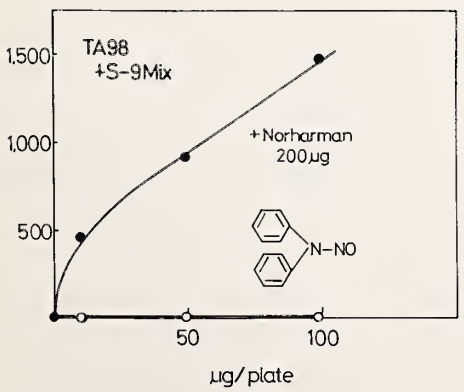
TEXT-FIGURE 3.—Comutagenic effect of norharman on aniline and *o*-toluidine.

only in its presence, it was named a "comutagen." Carcinogenesis can be divided into two stages: an initiation stage closely related to the mutational event and a promotion stage related to phenotypic changes. Comutagens such as norharman should be considered as "coinitiators" in the carcinogenic process.

Yellow OB, which was carcinogenic in rats but not mutagenic when alone, was mutagenic in the presence of norharman (*63*). Although the mechanism of the comutagenic action of norharman is unknown, two possibilities can be considered. One is that it is due to the intercalation of norharman into double-stranded DNA, which was demonstrated by changes in fluorescence and absorption of a solution of norharman when DNA was added (*64*). Digestion by DNase and ligation by DNA-ligase of fd-phage in the presence of norharman proved that it intercalated into DNA and caused unwinding of the double strands of DNA (*64*). Another possibility is that the comutagenicity is due to an effect of norharman on metabolism: Norharman may either induce enhanced metabolic activation or reduced metabolic inactivation of compounds (*40, 41, 62*) or inhibit monoamine oxidase (*65*). Because the comutagenic action of norharman has mainly been demonstrated with aromatic amines, the latter possibility is the more likely one.

Recently, diphenylnitrosamine was demonstrated to be carcinogenic (*22*). For a long time, this compound was regarded as a noncarcinogen, and it was actually selected as a negative control substance for an international collaborative study in a short-term test (*66*). Diphenylnitrosamine was not mutagenic to *S. typhimurium* TA100 or TA98 with S-9 mix; however, when norharman was added to the mixture of diphenylnitrosamine, S-9 mix, and strain TA98, diphenylnitrosamine became mutagenic (text-fig. 4). Inasmuch as this nitrosamine is used as a hardening substance in the rubber industry, humans may be exposed to it. Also, aniline may be formed from diphenylnitrosamine metabolically and may then exert a mutagenic action in the presence of norharman. Phenylmethylnitrosamine and phenylethylnitrosamine, weakly mutagenic toward TA100, were mutagenic toward TA98 only in the presence of norharman. All these findings suggest that diphenylnitrosamine or phenylalkynitrosamine may yield a substance related to aniline which eventually produces a mutagenic substance(s) in the presence of S-9 and norharman.

Of course, an explanation other than the aniline pathway is possible. Further experiments are required to elucidate the mechanism of the norharman effect. Addition of norharman to the system in Ames' method makes it possible for one to detect carcinogenic aromatic amine compounds by this procedure.



TEXT-FIGURE 4.—Comutagenic effect of norharman on diphenylnitrosamine. Ordinate = No. of *his*$^+$ revertants.

REFERENCES

(*1*) SUGIMURA T, NAGAO M, KAWACHI T, et al: Mutagen-carcinogens in food with special reference to highly mutagenic pyrolytic products in broiled foods. *In* Origin of Human Cancer (Hiatt HH, Watson JD, Winsten JA, eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1977, pp 1561–1577

(*2*) NAGAO M, HONDA M, SEINO Y, et al: Mutagenicities of smoke condensates and the charred surface of fish and meat. Cancer Lett 2:221–226, 1977

(*3*) SUGIMURA T, KAWACHI T, NAGAO M, et al: Mutagenic principle(s) in tryptophan and phenylalanine pyrolysis products. Proc Jpn Acad 53:58–61, 1977

(*4*) KOSUGE T, TSUJI K, WAKABAYASHI T, et. al: Isolation and structure studies of mutagenic principles in amino acid pyrolysates. Chem Pharm Bull 26:611–619, 1978

(*5*) YAMAMOTO T, TSUJI K, KOSUGE T, et al: Isolation and structure determination of mutagenic substances in L-glutamic acid pyrolysate. Proc Jpn Acad 54B:248–250, 1978

(*6*) WAKABAYASHI K, TSUJI K, KOSUGE T, et al: Isolation and structure determination of a mutagenic substance in L-lysine pyrolysate. Proc Jpn Acad 54B:569–571, 1978

(*7*) YOSHIDA D, MATSUMOTO T, YOSHIMURA R, et al: Mutagenicity of amino-α-carbolines in pyrolysis products of soybean globulin. Biochem Biophys Res Commun 83:915–920, 1978

(*8*) YAMAIZUMI Z, SHIOMI T, KASAI H, et al: Detection of potent mutagens, Trp-P-1 and Trp-P-2, in broiled fish. Cancer Lett 9:75–83, 1980

(*9*) SUGIMURA T, NAGAO M: Mutagenic factors in cooked foods. CRC Critic Rev Toxicol 6:189–209, 1979

(*10*) AKIMOTO H, KAWAI A, NOMURA H, et al: Synthesis of potent mutagens in tryptophan pyrolysates. Chem Lett 1061–1064, 1977

(*11*) TAKEDA K, OHTA T, SHUDO K, et al: Synthesis of a mutagenic principle. Chem Pharm Bull 25:2145–2146, 1977

(*12*) TAKAYAMA S, KATOH Y, TANAKA M, et al: In vitro transformation of hamster embryo cells with tryptophan pyrolysis products. Proc Jpn Acad 53B:126–129, 1977

(*13*) TAKAYAMA S, HIRAKAWA T, SUGIMURA T: Malignant transformation in vivo by tryptophan pyrolysis products. Proc Jpn Acad 54B:418–422, 1978

(*14*) ISHIKAWA T, TAKAYAMA S, KITAGAWA T, et al: In vivo experiments on tryptophan pyrolysis products. *In* Naturally Occuring Carcinogens-Mutagens and Modulators of Carcinogenesis (Miller EC, Miller JA, Hirono I, et al, eds). Baltimore: Univ Park Press, 1979, pp 159–167

(*15*) KADA T, MORITA K, INOUE T: Antimutagenic action of vegetable factor(s) on the mutagenic principle of tryptophan-pyrolysate. Mutat Res 53:351–353, 1978

(16) YAMADA M, TSUDA M, NAGAO M, et al: Degradation of mutagens from pyrolysates of tryptophan, glutamic acid and globulin by myeloperoxidase. Biochem Biophys Res Commun 90:769–776, 1979

(17) NEGISHI T, HAYATSU H: The enhancing effect of cysteine and its derivatives on the mutagenic activities of the tryptophan pyrolysis products, Trp-P-1 and Trp-P-2. Biochem Biophys Res Commun 88:97–102, 1979

(18) NAGAO M, YAHAGI T, HONDA M, et al: Demonstration of mutagenicity of aniline and *o*-toluidine by norharman. Proc Jpn Acad 53B:34–37, 1977

(19) NAGAO M, YAHAGI T, HONDA M, et al: Comutagenic actions of norharman derivatives with 4-dimethylaminoazobenzene and related compounds. Cancer Lett 3:339–346, 1977

(20) WYNDER EL, HOFFMANN D: Certain constituent of tobacco products. *In* Tobacco and Tobacco Smoke. New York: Academic Press, 1967, pp 317–495

(21) YASUDA T, YAMAIZUMI Z, NISHIMURA S: Detection of comutagenic compounds, harman and norharman in pyrolysis products of proteins and food by gas chromatography–mass spectrometry. *In* The Proceedings of the Japanese Cancer Association. 37th Annual Meeting. Tokyo: Japan Cancer Assoc, 1978, p 18

(22) CARDY RH, LIJINSKY W, HILDEBRANDT PK: Neoplastic and nonneoplastic urinary bladder lesions induced in Fischer 344 rats and B6C3F1 hybird mice and by *N*-nitrosodiphenylamine. Ecotoxicol Environ Safety 3:29–35, 1979

(23) WAKABAYASHI K, NAGAO M, KAWACHI T, et al: Comutagenic effect of norharman with *N*-nitrosamine derivatives. Mutat Res 80:1–7, 1981

(24) KONDO S, ICHIKAWA-RYO H: Testing and classification of mutagenicity of furylfuramide in *Escherichia coli*. Jpn J Genet 48:295–300, 1973

(25) KADA T: *Escherichia coli* mutagenicity of furylfuramide. Jpn J Genet 48:301–305, 1973

(26) ONG T, SHAHIN MM: Mutagenic and recombinogenic activities of the food additive furylfuramide in eukaryotes. Science 184:1086–1087, 1974

(27) TONOMURA A, SASAKI MS: Chromosome aberration and DNA repair synthesis in cultured human cells exposed to nitrofurans. Jpn J Genet 48:291–294, 1973

(28) WILD D: Mutagenicity of the food additive AF-2, a nitrofuran, in *Escherichia coli* and Chinese hamster cells in culture. Mutat Res 31:197–199, 1975

(29) YAHAGI T, NAGAO M, HARA K, et al: Relationships between the carcinogenic and mutagenic or DNA-modifying effects of nitrofuran derivatives, including 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, a food additive. Cancer Res 34:2266–2273, 1974

(30) YAHAGI T, MATSUSHIMA T, NAGAO M, et al: Mutagenicity of nitrofuran derivatives on a bacterial tester strain with an R factor plasmid. Mutat Res 40:9–14, 1976

(31) WALKER GC: Inducible reactivation and mutagenesis of UV-irradiated bacteriophage P22 in *Salmonella typhimurium* LT2 containing plasmid pKM101. J Bacteriol 135:415–421, 1978

(32) MIYAJI T: Acute and chronic toxicity of furylfuramide in rats and mice. Tohoku J Exp Med 103:331–369, 1971

(33) IKEDA Y, MORIUCHI S, FURUYA T, et al: Interim report: Induction of gastric tumors in mice by feeding of furylfuramide. Tokyo: Food Sanitation Study Council, Ministry of Health and Welfare, 1974

(34) NOMURA T: Carcinogenicity of the food additive furylfuramide in fetal and young mice. Nature 258:610– 611, 1975

(35) YOKORO K, KAJIHARA H, KODAMA Y, et al: Chronic toxicity of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) in mice, with special reference to carcinogenicity in the forestomach. Gann 68:825–835, 1977

(36) SANO T, KAWACHI T, MATSUKURA N, et al: Carcinogenicity of a food additive, AF-2, in hamsters and mice. Z Krebsforsch 89:61–68, 1977

(37) TAKAYAMA S, KUWABARA N: The production of skeletal muscle atrophy and mammary tumors in rats by feeding 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide. Toxicol Lett 1:11–16, 1977

(38) KINEBUCHI M, KAWACHI T, MATSUKURA N, et al: Further studies on the carcinogenicity of a food additive, AF-2, in hamsters. Food Cosmet Toxicol 17:339–341, 1979

(39) NAGAO M, YAHAGI T, KAWACHI T, et al: Comutagenic action of norharman and harman. Proc Jpn Acad 53:95–98, 1977

(40) LEVITT RC, LEGRAVEREND C, NEBERT DW, et al: Effects of harman and norharman on the mutagenicity and binding to DNA of benzo[*a*]pyrene metabolites in vitro and on aryl hydrocarbon hydroxlyase induction in cell culture. Biochem Biophys Res Commun 79:1167–1175, 1977

(41) FUJINO T, FUJIKI H, NAGAO M, et al: The effect of norharman on the metabolism of benzo[*a*]pyrene by rat-liver microsomes in vitro in relation to its enhancement of the mutagenicity of benzo[*a*]pyrene. Mutat Res 58:151–158, 1978

(42) AMES BN, MCCANN J, YAMASAKI E: Method for detecting carcinogens and mutagens with *Salmonella*/mammalian microsome mutagenicity test. Mutat Res 31:347–363, 1975

(43) SUGIMURA T, NAGAO M: Modification of mutagenic activity. *In* Chemical Mutagens. Principles and Methods for Their Detection (de Serres FJ, Hollaender A, eds). New York: Plenum Press, 1980, pp 41–60

(44) SUGIMURA T, KAWACHI T, NAGAO M, et al: Genotoxic carcinogens and comutagens in tryptophan pyrolysate. *In* Biochemical and Medical Aspects of Tryptophan Metabolism (Hayaishi O, Ishimura Y, Kido R, eds). Amsterdam: Elsevier/North-Holland, 1980, pp 297–310

(45) Carcinogenesis Testing Program, National Cancer Institute: Bioassay of aniline hydrochloride for possible carcinogenicity. DHEW Publ No. (NIH)78–1835. Bethesda, Md: Natl Cancer Inst, 1978

(46) YOSHIDA T, SHIMAUCHI T, KIN C: Experimentalle Studien uber die Entwicklung des Harnblasentumors. I. Gann 35:272–274, 1941

(47) NAGAO M, SUGIMURA T, MATSUSHIMA T: Environmental mutagens and carcinogens. Annu Rev Genet 12:117–159, 1978

(48) COMMONER B, VITHAYTHIL AJ, DOLARA P, et al: Formation of mutagens in beef and beef extract during cooking. Science 201:913–916, 1978

(49) KASAI H, NISHIMURA S, NAGAO M, et al: Fractionation of a mutagenic principle from broiled fish by high-pressure liquid chromatography. Cancer Lett 7:343–348, 1979

(50) PARIZA NW, ASHOOR SH, CHU FS, et al: Effects of temperature and time on mutagen formation in pan-fried hamburger. Cancer Lett 7:63–69, 1979

(51) SPINGARN NE, WEISBURGER JH: Formation of mutagens in cooked food. I. Beef. Cancer Lett 7:259–364, 1979

(52) KASAI H, NISHIMURA S, WAKABAYASHI K, et al: Chemical synthesis of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a potent mutagen isolated from broiled fish. Proc Jpn Acad 56B:382–384, 1980

(53) UYETA M, KANEDA T, MAZAKI M, et al: Assaying mutagenicity of food pyrolysis products using the Ames' test.

In Naturally Occurring Carcinogens-Mutagens and Modulators of Carcinogenesis (Miller EC, Miller JA, Hirono I, et al, eds). Baltimore: Univ Park Press, 1979, pp 169–176

(*54*) YAMAGUCHI K, ZENDA H, SHUDO K, et al: Presence of 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole in casein pyrolysate. Gan 70:849–850, 1979

(*55*) TOHDA H, OIKAWA A, KAWACHI T, et al: Induction of sister-chromatid exchanges by mutagens from amino acid and protein pyrolysates. Mutat Res 77:65–69, 1980

(*56*) HASHIMOTO Y, SHUDO K, OKAMOTO T: Structural identification of a modified base in DNA covalently bound with mutagenic 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole. Chem Pharm Bull 27:1058–1060, 1979

(*57*) _____: Metabolic activation of a mutagen, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole. Identification of 2-hydroxyamino-6-methyldipyrido[1,2-*a*;3′2′-*d*] imidazole and its reaction with DNA. Biochem Biophys Res Commun 92:971–976, 1980

(*58*) NEBERT DW, BIGELOW SW, OKEY AB, et al: Pyrolysis products from amino acids and protein: Highest mutagenicity requires cytochrome $P_{1\text{-}450}$. Proc Natl Acad Sci USA 76:5929–5933, 1979

(*59*) ISHII K, ANDO M, KAMATAKI T, et al: Metabolic activation of mutagenic tryptophan pyrolysis products (Trp-P-1 and Trp-P-2) by a purified cytochrome P-450-dependent system. Cancer Lett 9:271–276, 1980

(*60*) TSUDA M, TAKAHASHI Y, NAGAO M, et al: Inactivation of mutagens from pyrolysates of tryptophan and glutamic acid by nitrite in acidic solution. Mutat Res 78:331–339, 1980

(*61*) ARIMOTO S, OHARA Y, NAMBA T, et al: Inhibition of the mutagenicity of amino acid pyrolysis products by hemin and other biological pyrole pigments. Biochem Biophys Res Commun 92:662–668, 1980

(*62*) NAGAO M, YAHAGI T, SUGIMURA T: Differences in effects of norharman with various classes of chemical mutagens and amounts of S-9. Biochem Biophys Res Commun 83:373–378, 1978

(*63*) SUGIMURA T, NAGAO M, MATSUSHIMA T, et al: Recent findings on the relation between mutagenicity and carcinogenicity. Nucleic Acids Res [Spec Publ] 3:s41–s44, 1977

(*64*) HAYASHI K, NAGAO M, SUGIMURA T: Interactions of norharman and harman with DNA. Nucleic Acids Res 4:3679–3685, 1977

(*65*) HO BT, MCISAAC WM, WALKER KE, et al: Inhibitors of monoamine oxidase. Influence of methyl substitution on the inhibitory activity of beta-carbolines. J Pharm Sci 57:269–274, 1968

(*66*) DE SERRES FJ, SHELBY MD: Recommendation on data production and analysis using the *Salmonella*/microsome mutagenicity assay. Environmental Mutagenesis 1:87–92, 1979

Discussion I [1]

T. Sugimura: The session is now open for questions concerning the paper given by Dr. Dybing.

J. Weisburger: I would like to comment on the phenacetin part of Dr. Dybing's report because this aspect of the study is most important. Some time ago there was an exchange of letters in *Science* relative to the question of phenacetin carcinogenicity. While I was at the National Cancer Institute, we had one program during which we tested the aspirin, phenacetin, and caffeine tablets in animal models in the Bioassay Program and found it to be negative, as I remember.

Yet in the Japanese studies directed by Dr. Ito in which 1.5 and 2.5% phenacetin were fed to rats, it was definitely active in cancer of the urinary bladder. The data that Dr. Dybing reported from Sweden, Switzerland, and other places where abuse of phenacetin took place, in particular among employees of the dynamite factories where people had nitroglycerin-induced headaches, kilograms of phenacetin were consumed in about 10 years among the abusers.

Dr. Williams and I reviewed the data on phenacetin and it will eventually be published.

The point I wish to make is that animal tests reliably anticipate or confirm human data, i.e., high level exposure in animals and in man of those monocyclic arylamines lead to cancer; I wish to emphasize that there are no data whatsoever on low-level consumption. I think Dr. Dybing showed that in the United States production is high, but, as far as we know, there is no abuse, in general, of phenacetin. With regard to ineffective levels in animals, no other tests in animals were positive except Dr. Ito's when 1–2% was fed. As far as we know, no amount of phenacetin, except in abusers, led to disease.

Later, we will discuss this aspect a little more. Perhaps we can agree on some dose–response relationships. We must do much more research in this area, and even with mutagenic and carcinogenic arylamines, like phenacetin, perhaps one could find "no-effect" levels.

C. King: The phenacetin studies of Johannsen have shown that when 0.535% phenacetin was given to Sprague-Dawley rats, breast cancer and ear duct tumors were induced, but the incidence was considerably lower than that shown by the studies in Japan.

J. Weisburger: Well, those results seem to be in about the same ball park.

King: It is still lower. Furthermore, the definition of abuse in Sweden that was used was 1 g/day for a long period. That is the maximum recommended dose for many formulations presently available over the counter, without prescription, in this country.

There is a situation in Adrian, Michigan, not related to polychlorinated biphenyls, that many of you may be interested in, and that is the production of methylene-bis-*o*-chloroaniline (an aromatic diamine related to dichlorobenzidine, which has been used in many polymers) has reached a million pounds a year since 1971.

Since early 1979, investigators found that the entire area surrounding that factory is now heavily contaminated. This area comprises 7 square miles, includes most of the town of Adrian, and has about 40,000 to 50,000 residents. The workers and their spouses and children are excreting the unmetabolized compound, for which no good metabolic studies have been done. Perhaps only as little as 0.05% is excreted unchanged in rats. This unchanged chemical in the urine is the source material and base for many studies.

The investigators estimate that the body burden may be about 4 mg/day. The compound is carcinogenic in mice, rats, and dogs. The lowest dose thus far tested in rats is carcinogenic; a dose-related response was observed even in mammary tumors in male rats, which is quite extraordinary.

I think that anyone who has constructive ideas of what could be done to help alleviate that situation should notify the authorities. I think they would be most appreciative, particularly because they do not know at this point what to use or how to decontaminate. They have a large lagoon in which the level of this compound in the sludge is 0.2%. That sludge goes directly into the sewer system and the Raisin River, which is then used downstream for the water supply. The problem is acute in the sense of long-term carcinogenicity, although the people are not sick now.

T. Sugimura: Does anyone want to comment on that particular point?

J. Weisburger: I find that situation a total disaster. It is inexcusable that, in this decade, anybody would manufacture something that is a likely carcinogen. As you know, we had included methylene-bis-*o*-chloroaniline in the National Cancer Institute program, and researchers at E. I. du Pont de Nemours & Co., Inc., did also. That anybody would manufacture under such dramatically poor conditions, without disposing of the waste by incineration, is difficult to understand.

Unidentified participant: Dr. Sugimura, you are finding an increasing number of compounds that are mutagenic. I expect we will find that many of these will lead to cancer, but some will not. How does one of these mutagens that does not cause cancer react in a promotional system or in cocarcinogenesis with a known strong carcinogen? In other

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; 2-FA = 2-fluorenamine.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

words, have we found a pure initiator outside the skin system?

Sugimura: I think it is better to respond to your question later, after we finish the discussion of the previous two papers. Does anyone wish to comment on Dr. Dybing's paper?

W. Lenk: From which data do you infer the importance of the *N*-hydroxyphenacetin you have in the table? Are these studies in vivo or in vitro?

E. Dybing: Do you mean concerning the mutagenicity?

Lenk: You presented a table on the metabolism of phenacetin. I am aware of the results of the work of Hinson and Jollow. They could prove N-hydroxylation of phenacetin only with methylcholanthrene-treated or stimulated hamster microsomes, but it was never detected in urine. Is that really important? Which of your data influenced you so that you put *N*-hydroxyphenacetin in this major position?

Dybing: I tried to give an overview of the possible metabolic routes and did not intend to imply that this was a major pathway for phenacetin.

J. A. Hinson: We showed that phenacetin was an in vitro metabolite when we used hamster liver microsomes; we did not indicate that it was an in vivo metabolite. The in vivo metabolite we showed was *N*-hydroxy-*p*-chloroacetanilide, a metabolite of *p*-chloroacetanilide.

Recently, we have shown that *N*-hydroxyphenacetin can be further metabolized by de-ethylation. Once it is formed, you can have two competing pathways in vitro. You can have de-acetylation to produce the hydroxylamine or you can have de-ethylation to produce *N*-hydroxyacetaminophen.

Sugimura: To return to your comment on phenacetin, the phenacetin is not activated at all by rat liver microsomes, to my knowledge, even in the induced one. The microsome can easily activate phenacetin in hamsters. Dr. Bartch of the International Agency for Research on Cancer did this type of experiment.

S. Nelson: I think that Stewart McClain has done some studies recently with isolated rat hepatocytes in which he actually detected the formation of *N*-hydroxyphenacetin from phenacetin. When he administered *N*-hydroxyphenacetin to these isolated hepatocytes, he found little recovery of the hydroxy derivative, which indicated its considerable metabolism before it is released.

J. W. Gorrod: With regard to Dr. Clayson's early history of the subject, I think it is interesting that one talks about the German and the British chemical industries. Workers were exposed to huge amounts of toxicity. In fact, the people in the chemical industry were actually called by the nickname of "blueboys," because they literally had high levels of methemoglobinemia. At one level, we are talking about people with massive exposure, and on another, about people on chlornaphazin (about 200 g). Even then, only 4 or 5 got tumors.

In Rehn's original observation, only 3 papillomas were observed in the German chemical industry, and yet dozens of people were exposed.

Now we are talking about the possibility of trace amounts in foodstuffs. We have really stretched the limits of imagination of human exposure to this stuff, e.g., when we discuss plate tests of 2 μg 2-FAA.

Dr. Weisburger is right in talking about levels to which people are actually exposed. I wonder just how much hair dye a person actually gets exposed to and how much is absorbed? How much exposure do hairdressers, who presumably wear rubber gloves, receive?

The work on 4-aminobiphenyl was different. Thirty-five percent of those workers actually had urinary bladder cancer, i.e., those who had been exposed for 15 years or more. That was a real problem.

In a way, we are now scraping the barrel to find excuses. Are we really going the right way?

When we discussed the nitrogen compounds in the atmosphere, we focused on aliphatic amines, and here we are supposed to be concerned more with the aromatic amines. Is there a list of known aromatic amines? Monomethyl aniline does form nitrosamines, and they are certainly carcinogenic. We ought to pay a little bit of attention toward those and include the aromatic heterocyclic compounds. Dr. Clayson did not mention that β-naphthylamines are also reported as being present in tobacco smoke, and refinery workers have shown extremely low levels.

We have seen the range of 3 papillomas from massive exposure in the human population to micro exposure. We must be careful in our projections.

Trimethylamine is an end product of nitrogen metabolism in man and particularly in fish. Most of us excrete enough trimethylamine during the day to expect that we would have contributed to the environment, and certainly fish can as well. One wonders, in view of the fact that the triethylamine reaction goes on continuously, why more of it is not found in the environment. Some of these problems are difficult to understand.

Sugimura: The first part of your comment was on the issue of threshold, was it not? Threshold is a difficult issue; it is not limited only to aromatic amine compounds. Risk estimation completely depends on the issue of threshold; to my knowledge, no solid data are available.

This is an important subject, but it would require a 3-day symposium. We are already giving large doses to animals in research, and we are dealing with equal amounts of human exposure most of the time. Actually, we have been exposed to tiny amounts of each chemical. I do not know what the total exposure from all chemicals would be.

Dybing: I want to add a comment regarding exposure to hair dyes. It has been estimated that after the dark shades of hair dye are used, the absorption is between 1 and 3% of the dose, so that the systemic exposure would be approximately 0.1–1.0 mg/kg per exposure.

P. Magee: What is the actual source of the nitrosamines in the atmosphere? I understand that some of them probably come from factories. Are there other sources of amines in the atmosphere, e.g., from natural sources in forests? Could these amines come from, specifically, the diethylamine, which is nitrosated to produce the diethylnitrosamine?

D. Lokensgard: Certainly, there are anthropogenic sources of amines as well as natural emission from swamps and from biologic degradation and natural processes. Combustion of fossil fuels often produces some amines, e.g., in exhaust from automobiles, but not a great deal.

Most of the exposure that would be worthy of monitoring would be from industrial activity, where amines are either produced or separated from an industrial waste stream and improperly disposed of or vented to the atmosphere.

E. Weisburger: Some have proposed that diethylhydroxylamine be dispersed in the atmosphere of cities like Los Angeles, where there is a great deal of smog, and I would like to hear the comments of Dr. Lokensgard on this subject. Is this really beneficial? Would it cut down on smog? Would it introduce new harmful elements into our atmosphere?

Lokensgard: The odor threshold for diethylhydroxylamine is about 0.5 ppm. It has been reported to be efficacious in suppressing radical chain reactions at pressures of approximately a torr, which is about 0.001. We have shown that at less than parts per million concentrations, it does indeed inhibit the formation of radicals and thus delays smog chemistry to a certain extent.

After diethylhydroxylamine is used up, just as does any other material containing hydrocarbons, it participates in smog chemistry and actually serves as fuel. Apparently, it would not be a good idea to try this out. I am unaware of any studies about the health effects of the material itself.

E. Weisburger: I had heard that they did some pilot experiments in Israel with this compound. Have you heard anything on that matter?

J. Scribner: I would like to return to the problem of risk with relatively weak compounds at common exposure. If one does calculations for compounds like hair dye components and assumes risk based on relative mutagenicities and the known risk for benzidine-induced bladder cancer in man, we find that the calculated expected incidence of tumors would be difficult to detect, unless one were to do a prospective study on the entire population of hairdressers. Yet the absolute number of people to develop tumors would still be a good deal more than we would consider acceptable. So I think we are led back, from a regulatory viewpoint, to having to depend on animal tests for making decisions in this realm.

R. A. Floyd: I would like Dr. Lokensgard to comment concerning the fertility of the soil around the site where the hair was sampled. Specifically, I am thinking about the possibility of conversion of nitrites or nitrates in the soil to atmospheric nitrogen, and also if the nitrous oxide that you were finding may be an intermediate in this process. Do you have any comments?

Lokensgard: Some farming is done near Riverside, but little is at the Clermont site where some measurements were taken. As I said, we do not know what the source of the nitrous acid is in these studies. It might be the result of local industrial emission, agricultural microorganisms in the soil, or acid rain deposition onto nitrite laden soil. We do not know.

Sugimura: May I answer the question now which was raised earlier? To my knowledge, a typical carcinogen, like benzopyrene or nitrosoguanidine, also has the activity of a promoter. Let us take as an example the flavanoids, compounds contained in edible plants such as fruit, which show more mutagenic activity than does benzopyrene, but their carcinogenicity is negligible. Although 2-FA is an extremely potent mutagen, its carcinogenic potency is weak. Therefore, 2-FA may lack a kind of promoting activity on the skin; sebaceous gland damage was not observed after this chemical was applied on skin. Most of the complete carcinogens with promoting and initiating activities do show degeneration in the sebaceous gland. Theoretically and practically, a compound can be a pure initiator or close to that state.

E. Miller: My first question is for you, Dr. Sugimura. If you take any one of your broiled or charred conditions and you consider the mutagenicity of the amounts of the hydrolysis products that you have separated and analyzed, like the various tryptophan derivatives with or without harman and norharman present, have you drawn any kind of a balance sheet that tells you whether those mutagens and comutagens come anywhere close to accounting for the activity you observe?

I also have a comment related to Dr. Gorrod's statements. Though I am convinced, as I think everyone else is, that we are in a position where we cannot talk about real thresholds, or whether a compound is or is not going to be active with some definite level as the borderline, nevertheless, I think we have to talk about practical thresholds, i.e., that the risk certainly decreases with a decrease in dose. Depending on their uses in practical necessary activities, we may require some chemicals. However, I would exclude hair dyes because I think we can all get along without dyeing our hair.

I think we may or will have to accept some risk. As both scientists and the public, we have to make this acceptance of risk known to the public because many people consider that something carcinogenic presents a real risk. We are not sure it is a real risk. I think we need to do better in our efforts toward educating the public about carcinogens.

Sugimura: May I answer your first comment? Actually, the comutagenicity of norharman was discovered during the purification when the norharman was removed, i.e., pyrolysis of tryptophan; suddenly, mutagenicity was greatly diminished. So we tried to combine once again the separated fraction and found a tremendous increase in recovery of mutagenicity.

In other words, when we are dealing with crude materials containing many substances, the activity does not mean too much; it may be enhanced or suppressed. Naturally, many interactions occur.

Generally, we cannot say that this is a carcinogen or this is a mutagen. People have become fearful of this kind of information, The point I would like to emphasize is that we have an inevitable amount of exposure to environmental mutagens and carcinogens, and we need to accept this concept.

We have background radiation: cosmic rays and radioactive isotopes from the air and from our bodies that we cannot avoid. Therefore, we must learn to deal with having a small amount of radiation.

Also, we have a similar situation with environmental mutagens (this is my personal biased opinion).

H-G. Neumann: I agree entirely that we have to pay attention to the possibility of practical thresholds, but after being involved with committees who have to make the de-

cisions, I must add that we then have to give acceptable threshold values. I am pessimistic that, for the time being, we can give any figures and say at what point this practical threshold should be indicated.

To answer the comment of Dr. Weisburger, I would say that with phenacetin, we have one good example of a biochemical explanation for a threshold, as you all know. There is the saturation of one metabolic pathway, and the binding to cellular macromolecules increases beyond that saturation point dramatically. I would agree that this could form a basis to enumerate a certain threshold and to say that below that saturation, the risk is considerably lower than beyond the threshold. I will provide these figures in my presentation.

We measured the dose dependence of the reaction of metabolites of dimethylaminostilbene and found that any parameters were strictly linearly correlated with the dose, down to exposures in the parts/billion region. That means that, after a dose of 25 ng/rat, which is really a low dose, binding not only in liver but also in any extrahepatic tissue is exactly correlated with the dose. We observed no deviation from this linear correlation.

Although I agree completely that there should be practical thresholds, at the moment and for most of the carcinogens, we are unable to provide exactly what those thresholds should be.

Another topic that we have so little information on is the synergistic effects of various drugs, which is something we really should study in the future.

A. Gregory: I understand what happened with you, Dr. Sugimura, when you found that 2-FA was mutagenic. You went ahead, did a bioassay, and found that 2-FA was carcinogenic. Then it was subsequently banned.

One of the things that we need to recognize here is that if we had waited for an epidemiology study to alert us of the carcinogenicity of 2-FA, we probably would still be waiting and maybe waiting into the next century to find something like this.

When we have waited until we find epidemiologic evidence, we have really waited too long. I am not denigrating epidemiologic evidence; when it is available, we should use it. If substances are carcinogenic in animals, we can reasonably assume that they are carcinogenic in humans.

The other point that I wanted to raise was that once we find a substance is mutagenic, what do we do? Dr. Sugimura gave us an excellent example; but it took time, did it not, Dr. Sugimura? It took time to get the bioassay done and to get the substance prohibited.

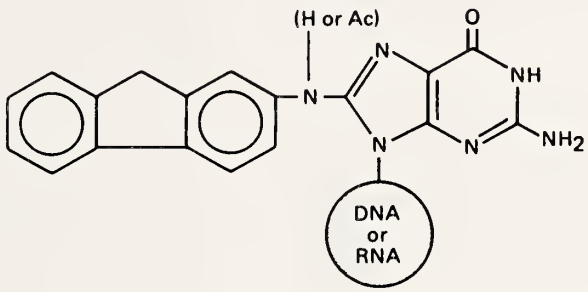
I will give you another good example of this. There is an extensively used herbicide called "propanil." All herbicides are pesticides, and all pesticides are herbicides. This substance will get rid of barnyard grass, which is a common contaminant in the seeding of rice. On the way back from Monte Carlo, I happened to be sitting next to a farmer, who is just starting to raise large amounts of rice. I asked him what kind of herbicide he used, and he said, "I use propanil." This firsthand information from a farmer sitting beside me on a plane coming back from Europe is real evidence to me that it is used in large amounts.

We have extensive information on this compound and know that there are up to 2,000 ppm of tetrachloroazobenzene in this herbicide, and, that when applied, it is also converted to tetrachloroazobenzene by bacteria in the environment. Tetrachloroazobenzene is one of the most mutagenic substances that has ever been tested, but we really do not know what the next steps in the investigations and control should be.

What do you do when you find something is highly mutagenic? In the United States, we do not really have a program whereby such substances can be studied in depth quickly.

Sugimura: I am sure that many of you also wish to comment on this subject as I do, but our time for discussion is up.

Session II: Animal Studies: Carcinogenesis



Session Chairman: Helmut R. Gutmann

Discussion Chairman: Peter Magee

Mechanisms of Species, Strain, and Dose Effects in Arylamine Carcinogenesis[1, 2]

J. H. Weisburger *and* E. S. Fiala[3, 4]

ABSTRACT—Arylamines are important in studies of chemical carcinogenesis because they have induced cancer in man occupationally exposed to high levels. Numerous animal models exist in which these types of chemicals exhibit activity. Thus far, only guinea pigs, steppe lemmings, and the GR mouse strain have been resistant. N-Hydroxylation is the demonstrated biochemical activation step for arylamines to form the proximate carcinogens, i.e., hydroxylamine derivatives. In most instances, these derivatives require a second activation step to yield the active electrophilic reactant. These activation steps are distinct as a function of the organ affected and overall determine the sensitivity of a given species. There is some indirect evidence that for certain target organs and for specific chemicals ring-epoxidation may also be an activation process. Considerable differences in activity as a function of structure within the class of arylamines have been observed. The recently reported oncogenicity of aniline may not represent true genotoxic carcinogenicity but may be mediated by an indirect effect on the hematopoietic system. Such effects are also noted with other reliably carcinogenic arylamines, but, because of a precocious development and high incidence of tumors, the effects on the hematopoietic system are expressed to a lesser extent. Substitution with *o*-methyl or *o*-methoxy groups usually, but not always, increases carcinogenicity, and the relevant mechanisms require further study. The effect of longer chain alkyl groups is not known. With polycyclic arylamines, there are considerable differences in activity as a function of the position of the amine group on the ring system. In general, substitution in a β- or *p*-position yielded powerful, rapidly acting carcinogens in animal models, and, with some specific chemicals, in man. Substitution in an α- or in an *o*-position with respect to vicinal aryl rings shows no or low activity, chiefly because of low rates of N-hydroxylation, probably due to high rates of competing detoxification reactions. Differences in relative carcinogenicity between single-ring arylamines and polycyclic arylamines differ 2 to 3 log. Thus higher continuing exposure levels are required to elicit cancer with single-ring arylamines. A recent example is the carcinogenicity of phenacetin when taken by drug abusers in whom doses on the order of 10 kg yielded cancer, but occasional lower intakes apparently did not. This situation was mimicked in animal models: Continuous lifetime intake of levels higher than 1.25% in the diet were required to yield cancer, whereas lower levels failed to do so. With most arylamines, current data showed a steep slope dose–response curve, although more research is required on the possible low level interactions as a basis of risk evaluation and rational disease prevention.—Natl Cancer Inst Monogr 58: 41–48, 1981.

Aromatic amines belong to a class of chemicals demonstrated as important causal agents for cancer in man as a result of his occupational exposure. The classic, historic cases described at the end of the last century by Rehn, which he termed "aniline cancers," have unfortunately been followed by other cases extending up to the present time (*1–6*). Some of the high bladder cancer incidence in areas such as New Jersey is accounted for by exposure, decades ago, to certain aromatic amines which have not been manufactured there for about 25 years. Thus it is important for one to understand the nature of carcinogenic processes and to have tools available to detect potential carcinogens and minimize the risk of disease which may have protracted latent periods. Virtually all occupational exposures to aromatic amines have led to cancer in the renal pelvis and the urinary bladder.

Here we review the question of species and strain specificities in relation to structure of arylamines and provide the background of the underlying mechanisms.

The arylamines known to cause cancer in man were derivatives of polycyclic aromatic amines like 2-NA or of biphenyl derivatives like 4-biphenylamine and its derivatives (*3*, *4*). During the last 50 years, numerous structures were tested for carcinogenic activity, and a number of arylamines were highly carcinogenic in animal models. Until recently, few tests were conducted with single-ring amines, and much of the earlier data base includes chemicals with more than 1 benzene ring. The reader is referred to some of the many reviews available (*2*, *7*). In addition to aromatic amines, we need to include the carcinogenic azo dyes, inasmuch as most of these chemicals are active because of their arylamine functional grouping (*8*, *9*). Much less is known about heterocyclic amines. This field is of increasing potential importance since the discovery by Sugimura's

Abbreviations: 2-NA = 2-naphthylamine; BPA = biphenylamine; OH = hydroxy; 2-FAA = *N*-2-fluorenylacetamide; NCI = National Cancer Institute; 2-FA = 2- fluorenamine; SCD = standard cumulative dose, defined as moles of carcinogen per kilogram of diet multiplied by the number of weeks of feeding.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported in part by Public Health Service grants CA15400, CA24217 (through the National Large Bowel Cancer Project), CA26395, and CA12376 from the National Cancer Institute, and OH00611 from the National Institute of Occupational Safety and Health.

[3] American Health Foundation, Naylor Dana Institute, Valhalla, New York 10595.

[4] We thank Mrs. C. Horn and Mrs. H. Sacks for editorial services.

group (*10*) that this kind of chemical may be causal in the development of human cancers.

In the last 20 years, a beginning has been made in the study of the mode of action of arylamines as carcinogens, (*7, 8*). The Millers discovered, as did Kiese and Uehleke independently, and almost simultaneously the Weisburgers, that arylamines could undergo biochemical N-oxidation. With carcinogenic arylamines, this reaction resulted in the formation of the proximated carcinogenic N-OH-derivative. Most of those studies were conducted with the N-acyl-derivative 2-FAA and with the prototype azo dye 4-dimethylaminoazobenzene. This proximate N-OH intermediate required additional conversion to an electrophilic reactant; certain of these reactions have been discussed at this Symposium.

However, in the intervening years, the theoretical concepts in cancer causation have been clarified with the realization that cancer is most likely the result of a somatic mutation through carcinogen-induced changes in the genetic apparatus, especially DNA. Having taken this view, researchers could assume a relationship with bacterial mutagensis (*11*) which, in turn, provided a powerful tool for their studying the mechanisms of action of carcinogens at the molecular level. At the same time, ultimate carcinogens could be detected by their property of inducing mutations in bacteria [the Ames Test (*12*)] or in hepatocytes [the Williams Test (*13*)].

Most arylamines are active in indicator systems typical of frameshift mutants like *Salmonella typhimurium* TA1538 or TA98. Polycyclic aromatic hydrocarbons are less active with these indicator systems and more so in indicator systems that reflect base-pair mutations, in which recent developments indicated that the ultimate electrophilic reactant is a specific dihydrodiol epoxide. Certain of the aromatic amines which were carcinogenic, sometimes at unconventional target sites, such as the skin, subcutaneous tissue, and even colon, are mutagenic in the frameshift and base-pair mutation indicator systems. These results are consistent with the interpretation that certain arylamines induce cancer not only through an active intermediate obtained by metabolism on the nitrogen but possibly by epoxidation in the benzene ring. Validation of this concept by more research is essential. When calling attention to this possibility 20 years ago, we used a different line of reasoning (*14*).

STRUCTURE-ACTIVITY RELATIONSHIPS

Valuable older studies conducted under comparable conditions that involved feeding or otherwise administering equimolar amounts of chemicals, sometimes for limited periods of time, provided a fundamental base of information (*2, 15*). Although the concepts derived are unquestionably still valid, one must realize that current methods of carcinogen bioassay involving administration of the test compound at the maximally tolerated dose (equitoxic, not equimolar) for a lifetime may modify these concepts to some extent (*16–19*). Nonetheless, the basic structure–activity correlations (table 1) have also found support at the level of molecular mechanisms (*8, 20–22*). The question may arise whether more prolonged, high-level testing that yields certain other data should not receive a thorough mechanistic investigation before conclusions are drawn on the risk to man based solely on such bioassay findings. For example, in a test series of simple arylamines, we used aniline as a negative control. Heretofore, aniline was considered inactive as a result of several studies on it or acetanilide (*2, 5*). However, thorough testing showed that male rats fed high levels of aniline for a lifetime had moderate incidence of splenic hemangiosarcomas compared with untreated control rats. Female rats had borderline responses with the same type of lesion, but mice fed higher doses were negative. Why would this be so? Can one deduce that aniline is a carcinogen, and more importantly, a carcinogenic risk to man as is, e.g., 4-BPA?

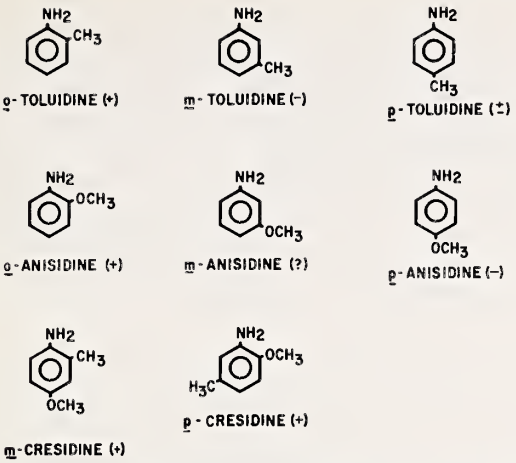
TABLE 1.—*Structure specificity of aromatic amines*

Polynuclear *α*-arylamines are not carcinogenic because they N-hydroxylate poorly.
N-Acetyl derivatives are equally or more active except with *o*-alkyl substitution.
Single-ring aryl compounds are less active than polynuclear arylamines.
o-Methyl substitution often, but not always, increases activity.
Mutagenicity in the Ames system often, but not always, correlates with rodent carcinogenicity.
Activity in the Williams system almost always correlates with rodent carcinogenicity.

Single-Ring Arylamines

For many years we have been concerned with the question of the possible carcinogenicity of single-ring arylamines. While the senior author was associated with Dr. H. P. Morris, we (*23*) discovered that a prototype, 2,4,6-trimethylaniline (mesidine), induced hepatocellular carcinoma in rats. This finding was extended by Ito et al. (*24*), who also found that 2,4-diaminotoluene caused such tumors. Therefore, we initiated a systematic program of research as part of the NCI Bioassay Program. Chronic high-level intakes of aniline induced subcutaneous sarcomas in moderate yield through ill-defined mechanisms. Hemangiosarcomas were noted only in the spleens of male rats but not in male and female mice. These tumors may have stemmed from the well-known effect of aniline and related compounds on the hematopoietic system described so well by Kiese (*25*) and Uehleke (*26*).

Among the toluidines, the *o*-isomer was definitely carcinogenic, but the *m*-isomer was negative, and the *p*-isomer, with a questionable effect, may well be negative (text-fig. 1). Among the isomers of the anisidines (or methoxyanilines), the ortho is definitely active and more so than the corresponding toluidine, the para is negative, and no data are available on the meta. With cresidines, the *p*-isomer, which has an ortho-methoxy substitution, is more active than the meta with its ortho-methyl substitution. It would seem, therefore, that ortho-methoxy leads to a higher carcinogenic activity than does the ortho-methyl.



TEXT-FIGURE 1.—Carcinogenicity (indicated *in parentheses*) of methyl- and methoxy-substituted aniline derivatives in B6C3F$_1$ mice and/or F344 rats. *Note* the carcinogenicity of ortho-methyl- or ortho-methoxy-substituted anilines, and the negative effect of meta- or para-substituted derivatives.

Only the *o*-isomer of phenylene diamines exhibited some activity, but the meta and para compounds were inactive. Chloro substitution, as in 4-chloro-*o*-phenylenediamine, increased carcinogenicity especially for the urinary bladder in rats. The correlation between mutagenicity in the Ames system and carcinogenicity in this series of compounds is poor. Lack of detailed knowledge of the relevant mechanism is a gap that should be filled.

Among the toluenediamines, only the 2,4-isomer was active. As noted by Ito et al. (*24*), this chemical caused liver tumors in male rats, but the new NCI tests showed it also induced mammary gland tumors in female rats. Thus the monocyclic amine has properties similar to those of the polycyclic arylamines. The 2,5- and 2,6-isomers, on the other hand, were not carcinogenic, and thereby testified to the importance of chemical structure in carcinogenesis. The importance of a 2,4-diaminotolyl structure is underlined by the fact that methyl could be replaced by methoxy or ethoxy and maintain carcinogenicity, albeit with a shift in target organ, the thyroid, perhaps because such structures mimic tyrosine and thyroxine.

A para-substituted aniline, *p*-ethoxy-*N*-acetylaniline or phenacetin, has been suspected of being involved in renal and bladder cancer in persons who abuse the drug (*27*). The disease was diagnosed in patients seen in Sweden and Switzerland who had consumed kilograms of the drug. However, a number of animal tests with phenacetin were negative. At NCI, we initiated a test in which we used a mixture of aspirin, phenacetin, and caffeine to mimic more realistically the drug composition often used by people, but this was not effective in evoking a parallel of the human disease condition. However, Isaka et al. (*28*) recently recorded the results of a test in which diets containing 1.25 and 2.5% phenacetin were fed to rats for 18 months, and then the rats continued on a basal diet for another 6 months. At the lower dose level, tumors of the nasal passages were the main lesions; at the higher dose, tumors of the nasal cavity and, more importantly, tumors of the kidney and urinary bladder were observed. Thus it seems that the condition mimicking the human high-level *abuse* was reproduced accurately in animals (*29*). Apparently, with this kind of agent which might normally be classified as a weak carcinogen, there may be a limit with respect to effective dosages leading to cancer in man as well as in animals. This may be an instance when occasional intake of moderate amounts may have no untoward effect, whereas high intakes in animals and in man may induce cancer. That the animal test accurately mimicked a human situation is an important finding in the interpretation of the results of other bioassays with this and perhaps other classes of carcinogens. Thus even a "weak" carcinogen which affects man consuming only large quantities produces a high incidence of specific cancers in experimental animals, provided that long enough periods of feeding and observation are used. These are the same conditions we developed for a standard bioassay (*16, 19*).

The series based on *o*-toluidine yields a similar picture of increased carcinogenicity as more aryl rings, either anellated or in a straight line, are added to the basic molecule as in 3-methyl-2-NA or in the series of 3-methyl-4-BPA derivatives, first found active by Walpole (*30*). Whereas *o*-toluidine is a weak carcinogen in the rat bladder, *o*-methyl-2-NA and 4-BPA derivatives cause cancer not only in the urinary bladder but also in the intestinal tract, mammary gland, ear duct, and salivary gland. Ortho-methyl substitution also serves to convert the α-substituted 1-aminoanthraquinone to weak but definitely active carcinogens through mechanisms which require definition.

Higher Homologs

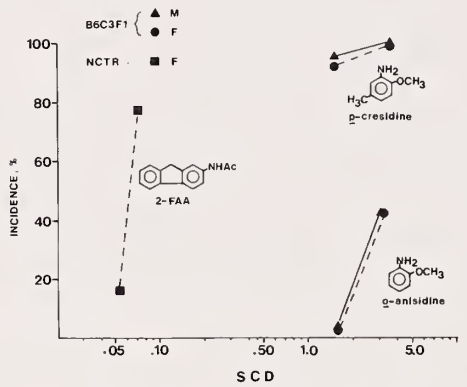
Whereas aniline has questionable carcinogenicity, 4-BPA is a potent carcinogen in several animal species and in man. Likewise, 2-NA, 2-FA, 2-anthramine, and other 2-substituted polycyclic arylamines with anellated ring systems are powerful carcinogens in animals and have induced cancer in man in some instances. Such chemicals are also uniformly active as mutagens in the Ames system and require biochemical activation. In contrast, neither pure 1-NA, 1-FA, and related compounds nor 2-BPA have been found to be carcinogenic; generally, these chemicals are also negative in the Ames and Williams tests (*12, 13*). To a great extent, the fact that they are not converted to the corresponding N-OH-derivative accounts for their lack of activity. The corresponding hydroxylamines are, however, mutagenic and carcinogenic.

STRUCTURE-EFFECTIVE DOSAGE

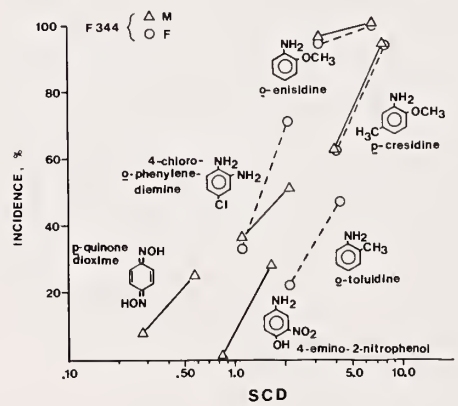
An additional important consideration when structure–activity relationships are compared is the question of the necessary dosage and length of administration (table 2). To evaluate such relationships, we found SCD useful as a parameter. The single-ring arylamines show an SCD index 100 to 500 times larger than polycyclic arylamines, and even then the latter compounds yield more cancers at various specific target organs more rapidly (text-figs. 2–5). This distinction, important theoretically with respect to the

TABLE 2.—*Dose-response studies on arylamines*

A single dose is rarely effective, in contrast to other carcinogens. The exception is the induction of mammary gland tumors in the sensitive female Sprague-Dawley rat, in which single doses of certain arylamines and diamines are effective.
Route of administration is not crucial, and feeding is customary.
With polynuclear arylamines, dietary levels of 50–800 ppm are usually effective in yielding various types of cancer quickly, often in less than 1 yr in rodents.
4-Dimethylaminostilbene requires less (5 ppm) and 2-NA more (up to 10,000 ppm).
Single-ring arylamines require up to 10,000 ppm for life.

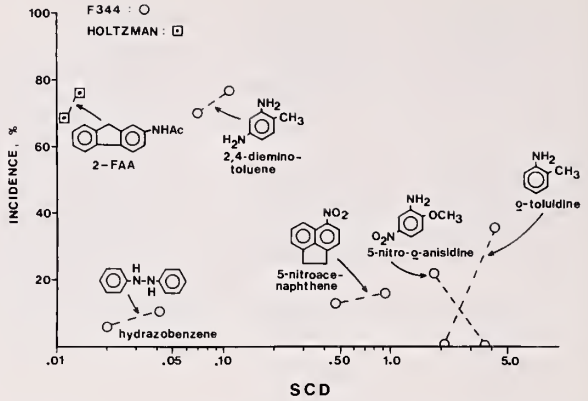


TEXT-FIGURE 2.—Comparison of potencies of arylamine carcinogens for the urinary bladder with the use of SCD in B6C3F$_1$ and NCTR mice. *Note* the appreciably lower potency of single-ring arylamines compared with the polycyclic ring amines.

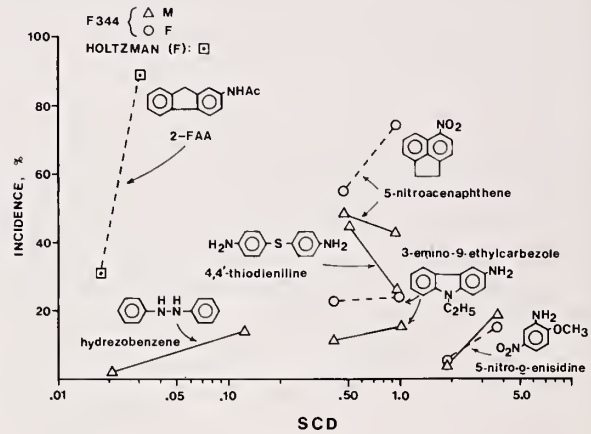


TEXT-FIGURE 3.—Comparison of potencies in F344 rats of arylamine carcinogens for the urinary bladder.

relevant mode of action, is also most important practically with respect to protecting the general public or specific groups such as workmen against the development of cancer. Carcinogenic activity with smaller doses of the polycyclic arylamines may reflect a higher rate of metabolic activation or the reverse, a lower rate of detoxification than occurs with the smaller molecules of the single aryl ring



TEXT-FIGURE 4.—Comparison of potencies of aromatic hydrazo, amino, and nitro derivatives in induction of mammary tumors in female F344 and Holtzman rats (*15, 45*).



TEXT-FIGURE 5.—Comparison of potencies of aromatic hydrazo, amino, and nitro derivatives in induction of ear duct tumors in F344 and Holtzman rats (*15, 45*).

type. These quantitative differences may not necessarily be reflected in the rapid in vitro tests such as mutagenicity assays in bacterial systems, e.g., the Ames test. Such systems do not have the same kind of quantitative activation–detoxification relationships and do not provide a conjugation–excretion mechanism inherent in the in vivo situation. In this respect, the Williams test may be more akin to the vivo system, inasmuch as certain detoxification enzyme systems are still present in the primary liver cell explants.

Consideration of the structure-activity relationships suggests two broad principles: 1) All other things being equal, arylamines with *o*-aryl grouping, either through a C–C–phenyl link (e.g., 2-BPA) or an anellated ring system (e.g., 1-NA) are usually considered as not being carcinogenic. The underlying reason seems to be an absence or at least a lower rate of the essential first metabolic activation step or N-hydroxylation. 2) Other arylamines with the amino groups in other parts of the ring are often carcinogenic (especially in polynuclear aromatic ring systems) and do undergo metabolic activation by N-oxidation. All other things being equal, the larger the aryl, the larger the mole-

TABLE 3.—*Species specificity of aromatic amines*

All species tested are sensitive to specific arylamine derivatives except the guinea pig and steppe lemming.
Insensitive species have low titers of N-hydroxylation enzyme systems or high titers of competing detoxification systems.
Within species, strain differences exist due partly to N-hydroxylation and partly to other activating systems to the ultimate carcinogenic metabolite.
Man is heterogeneous and exhibits individual sensitivity.

TABLE 4.—*Organ and tissue specificity of aromatic amines*

Polynuclear arylamines often affect the liver, bladder, mammary gland, and ear duct.
o-Methyl polynuclear arylamines additionally cause cancer in the intestines.
Nitro compounds also induce squamous tumors in the forestomach.
Methylanilines can cause subcutaneous, liver, bladder, and mammary gland tumors, as well as hemangioendotheliomas.

cular size and the multiplicity of benzene rings (2–4 rings), the more likely a given chemical is carcinogenic. Whereas larger molecules tend to be insoluble and therefore perhaps less active, smaller molecules require much higher doses to elicit an effect; the underlying biochemical mechanisms may be different and need more research.

SPECIES AND STRAIN DIFFERENCES

Some aromatic amines have demonstrated carcinogenic effects in man exposed at his workplace. Thus far, no nonoccupational environmental exposures to aromatic amines have demonstrated carcinogenic effects in man.

Different species have been exposed to certain aromatic amines. The most studied compound, 2-FAA, exhibited carcinogenic activity in all species except the guinea pig and steppe lemming (*31*). Rats, mice, and hamsters of different strains have responded positively but not always in the same target organ (table 3). Dogs, cats, and rabbits also gave evidence of cancer. Generally, a responsive species will have demonstrated a capacity to convert the arylamine to an N-OH-derivative, the proximate carcinogen, and the nonresponsive steppe lemmings and guinea pigs do so only to a small extent or not at all.

With certain species, there are distinctions in the quantitative aspects as a function of strain. Likewise, man exhibits diverse capability to H-hydroxylate. We proposed years ago that in areas of potential exposure to aromatic amine carcinogens, a screening system be used for the selection of individuals with the lowest capability to convert aromatic amines to the N-OH-derivative (*32*). It is likely that such individuals would be at low risk.

EFFECT OF STRUCTURE ON THE TARGET ORGAN

In sensitive mouse strains, most arylamines induce liver tumors. In addition, certain specific aromatic amines also induce tumors in the urinary bladder, and some yield hemangioendotheliomas (table 4). Under some specific conditions and as a function of mouse strain and structure of arylamine, other kinds of neoplastic lesions have been induced.

In hamsters, the liver and the urinary bladder are likewise the principal target organs, but under some conditions, particulary with inbred strains, other tumors, such as those of the mammary gland, also develop (*33*).

With certain of the classic arylamines, e.g., 2-FAA, various tumors are seen, principally those of the liver, urinary bladder, and ear duct, and cancer of the mammary glands is often noted in certain strains of female rats.

Some chemicals have an apparent and more restricted diversity of target organs. Under the standard protocols of testing used by the Millers and their research group, 4-acetylaminobiphenyl was virtually inactive in male rats and primarily caused breast cancer in female rats. 4-Dimethylaminostilbene yields ear duct tumors almost exclusively. The N-OH-derivatives of these chemicals appear more specific, and thus it is presumed that they affect these target organs. On the other hand, skin tumors are rarely induced by aromatic amines as such. However, when the skin of mice fed 2-FAA was treated with croton oil, skin tumors resulted [table 5; (*34*)]. More tumors were seen when the parent FAA, rather than the N-OH-derivative, was administered. Therefore, skin cancer induction may involve a proximate and ultimate carcinogen different from the N-oxidation product, such as an epoxide. In our studies of the metabolism of acetanilide, a 3,4-epoxide intermediate was postulated (*35*). An intermediate of this type may also account for the skin tumors induced with 2-anthramine in rats (*14*).

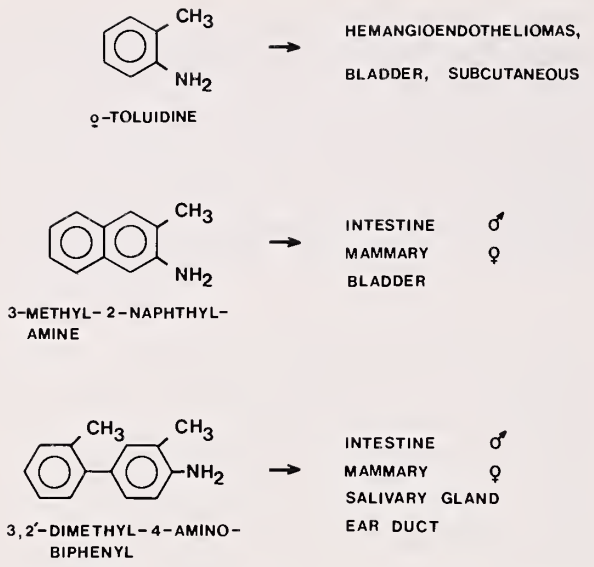
The general principles discussed above on molecular size versus effectiveness are also true when the actions for each kind of target organ and species are considered. Thus smaller molecules require higher dose levels than do the larger ones for tumor induction in the mammary glands of female rats, urinary bladder in rats and mice, or in the ear ducts of male or female rats.

Elsewhere we discussed the interesting and as yet imperfectly understood property of ortho-methyl substitution in increasing the potency of arylamines and of altering their

TABLE 5.—*Skin tumors in mice fed 0.05% 2-FAA or 0.054% N-OH-2-FAA and treated with 0.05 ml of 0.5% croton oil twice/wk during months 3–7* [a]

		Testing periods					
		4.5 mo		7 mo		9 mo	
		No. of:		No. of:		No. of:	
Carcinogen	No. of mice at 5 mo	Mice with tumors	Tumors	Mice with tumors	Tumors	Mice with tumors	Tumors
2-FAA	49	17	25	21	43	15	30
N-OH-2-FAA	43	4	4	12	19	11	24

[a] Data are from (*34*).

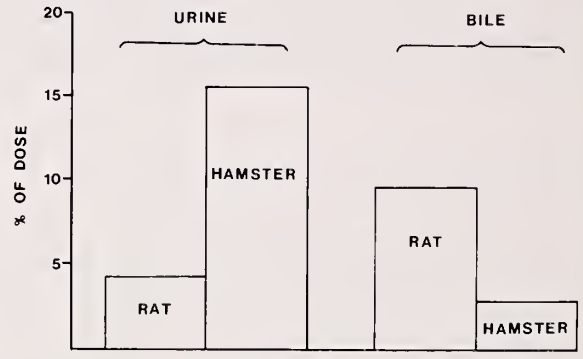


TEXT-FIGURE 6.—Target organs of ortho-methyl arylamines in rats. Ortho-methyl substitution generally leads to increased carcinogenicity. An exception seems to be *o*-tolidine (or 3,3′-dimethylbenzidine) which is less active than benzidine in tests conducted thus far (*see* text).

TABLE 6.—*Carcinogenicity of 3,2′-dimethyl-4-aminobiphenyl in male F344 rats and Syrian golden hamsters*

Site	Incidence, %[a]	
	Rat	Hamster
Large intestine	27	16
Small intestine	30	16
Urinary bladder	0	83
Ear duct	7	0
Skin	33	0
Subcutaneous	10	0

[a] 3,2′-Dimethyl-4-aminobiphenyl was given sc to 30 rats and 25 hamsters in 20-mg and 100-mg/kg doses, respectively, weekly for 37 wk.



TEXT-FIGURE 7.—Percent of dose of 3,2′-dimethyl-4-aminobiphenyl and/or metabolites excreted in the 24-hr urine or bile of male F344 rats and male Syrian golden hamsters after a single 75-mg/kg sc inoculation.

target organ (text fig. 6). *o*-Toluidine is certainly more powerful than aniline in animal models, and 3-methyl-2-NA and 3-methyl-4-BPA are more powerful carcinogens in different target organs than the corresponding amines not substituted with the ortho-methyl group. The possible exception to this statement may be the fact that 3,3′-dimethylbenzidine (tolidine) appears less carcinogenic then benzidine. Apparently 3,3′,5,5′-tetramethylbenzidine is not carcinogenic; therefore, it has been proposed as a useful, nonhazardous, laboratory reagent for various applications in clinical pathology and chemistry instead of benzidine.

Why ortho-methyl substitution has these effects is not yet known, but it is under active study in our Institute. The 3-methyl-4-BPA type of compounds usually induce cancer of the large intestine in certain rat strains and are therefore used as models to study this important cancer which is seen frequently in man (*36*). When given under identical conditions to hamsters, these chemicals primarily induce cancer in the urinary bladder; they do so less frequently in the intestinal tract (table 6). Whereas the urine of the hamster contains more metabolites than does that of the rat, the bile of the rat contains more metabolites than does the bile of the hamster, hence the correlation with the distinct biologic effects (text-fig. 7). Although the bile of the rat contains an N-OH-compound conjugated as glucuronide in sizable amounts, the same component is also present in similar amounts in hamster urine.

Therefore, the hamster should show more bladder cancer, whereas the rat would exhibit more intestinal neoplasia. However, in a recent series of experiments with hamsters that had intestinal enteritis, we found not only urinary bladder cancer but also a higher incidence of intestinal cancer (*37*). This higher incidence may be a model for ulcerative colitis which in man is a high-risk condition for colon cancer. We (*38*) postulated that a carcinogenic stimulus in this condition may be present in fried foods, especially fried meats or fish.

Studies with these compounds are of great contemporary interest because they do represent an approach to our understanding of the mechanism of action of agents that cause cancer in the intestine and the bladder, which are important target organs in the human. The relevance of this approach is further underscored by the discovery that certain pyrolysis products of tryptophan and other amino acids are heterocyclic amines, and intriguingly, certain of these have an ortho-methyl aryl, heterocyclic amine structure. The structure of the compounds in fried foods is probably not quite like the pyrolysis products of pure amino acids (Spingarn NE et al: Unpublished data; (*39*)]. Spingarn and Garvie (*40*) acquired some evidence that chemicals akin to those seen in the browning reaction, such as heterocyclic amines, may be involved. In our current investigations, we (*41, 42*) attempted to relate the occurrence of colon and other important cancers in man, e.g., cancers of the breast and prostate, to the higher intake of fried foods and meat by people who develop these diseases.

We discussed above the finding that skin tumors in mice were obtained when a promoting agent was applied after

they were fed a carcinogenic aromatic amine. Most of the larger molecular weight aromatic amines cause cancer at the most prevalent target sites, i.e. liver, ear duct, mammary gland, bladder, or intestinal tract in less than a year. On the other hand, the smaller, single-ring, carcinogenic arylamines rarely induce cancer is such a short time and with such low dose levels. Subcutaneous tumors are seen mainly when the animals are held for 2 years or in lifetime studies and a large amount of chemical is administered. In some instances, e.g., when *o*-toluidine was used, subcutaneous sarcoma was the main neoplastic reaction, although a few bladder tumors were also seen. With the polycyclic arylamines like 2-FA, tumors at injection sites after sc administration are rarely if ever observed. In an experiment decades ago [*see* (*5*)], a solution of pure 2-NA failed to induce subcutaneous sarcoma anywhere, although an older impure solution did so. Radomski (*6*) identified one product stemming from the oxidative decomposition of 2-NA that should be independently tested for oncogenicity.

The stability of the highly purified chemicals used in the NCI Bioassay Program during administration was continuously checked. Thus it is difficult to postulate that oxidative decomposition products accounted for the subcutaneous sarcoma induced. The higher incidence of skin tumors that mice fed *N*-2-FAA developed compared with those fed its *N*-OH-derivative (*34*) indicates that N-oxidation may not be the activation reaction for the occurrence of subcutaneous tumors. If one considers the fact that polycyclic aromatic hydrocarbons have shown a high potency in inducing subcutaneous sarcomas and that this most likely involved an intermediate ultimate carcinogen in the form of an epoxide (*43*), one can also postulate such an intermediate with the arylamines. It is not known whether an intermediate dihydrodiol is formed or whether the *o*-methyl amine function may play a role in directing and stabilizing epoxide formation. This consideration at this point applies mainly to the induction of subcutaneous tumors in mice or rats. Current research may show whether it applies also to the induction of intestinal tract cancer by certain of these compounds like the 3-methyl-4-BPA series. Our view is based on the fact that gut bacterial enzymes not only split any *N*-OH-metabolite conjugate entering the intestine by way of the bile, but they also remove the N-OH function (*44*).

CONCLUDING COMMENTS

Certain arylamines are demonstrated carcinogens for man, mainly in the urinary bladder. In the past, the simplest aromatic amine, aniline, or the derived acetanilide were considered as noncarcinogens. A careful evaluation of the latest test series with aniline conducted at the NCI indicated a small yield of subcutaneous tumors with high levels of aniline given in a lifetime study and may represent evidence of oncogenicity (*45*). However, this activity does not necessarily reflect a carcinogenic hazard in other conditions especially in extension to man. The tumors seen may have been the result of continuous long-lasting stress on the hematopoietic system and not that of direct genotoxic action. Study of the underlying mechanisms is imperative if we are to form the foundation for soundly based regulatory action to protect man. Smaller molecular weight arylamines have lower overall carcinogenic and oncogenic effects than do larger molecules, such as 1-NA or 2-BPA. When the amino group is alpha to an anellated ring position in a polycyclic arylamine, current views are that such chemicals are not carcinogenic because they cannot undergo the required biochemical N-hydroxylation reaction to a sufficient extent. Also, species like guinea pigs and steppe lemmings that cannot perform this activation step are not sensitive to arylamines. Because the human has a widely varying capability to perform this essential activation step, by extrapolation some individuals would be expected to show distinct sensitivity to arylamines. Ortho-methyl substitution in an arylamine system often, but not always, increases carcinogenicity and usually diversifies the target organs.

More research for better definitions of conditions leading to species and strain differences in overall specific arylamines and improved understanding of the relevant mechanism of action are essential. Mutagenicity tests in bacterial and mammalian systems are useful indicators in qualitative but not quantitative terms. More research is indicated for the provision of a basis for risk assessment with this class of compounds, especially because certain materials with heterocyclic arylamines might play an increasingly larger role in the context of potential human exposure. Such activities should form a rational basis for cancer prevention in man.

REFERENCES

(*1*) Shimkin MB, Triolo VA: History of chemical carcinogenesis. Some prospective remarks. Prog Exp Tumor Res 11:1–20, 1969

(*2*) Arcos JC, Argus MF: Chemical Induction of Cancer, vol 2. New York: Academic Press, 1974

(*3*) Shubik P: Forum: Occupationally-induced diseases. Introduction. Prev Med 5:226–227, 1976

(*4*) Clayson DB: Occupational bladder cancer. Prev Med 5:228–244, 1976

(*5*) _____: Chemical Carcinogenesis. Boston: Little, Brown, 1962

(*6*) Poupko JM, Hearn WL, Radomski JL: N-Glucuronidation of N-hydroxy aromatic amines: A mechanism for their transport and bladder-specific carcinogenicity. Toxicol Appl Pharmacol 50:479–484, 1979

(*7*) Clayson DB, Garner RC: Carcinogenic aromatic amines and related compounds. *In* Chemical Carcinogens, American Chemical Society Monogr No. 173 (Searle CE, ed). Washington, D.C.: Am Chem Soc, 1976, pp 366–461

(*8*) Miller JA, Miller EC: Perspectives on the metabolism of chemical carcinogens. *In* Environmental Carcinogenesis. Occurrence, Risk Evaluation and Mechanisms (Emmelot P, Kriek E, eds). Amsterdam: Elsevier/North Holland, 1979, pp 25–50

(*9*) Odashima S, Takayama S, Sato H, eds: Recent Topics In Chemical Carcinogenesis. Gan Monogr No. 17. Baltimore: Univ Park Press, 1975

(*10*) Nagao M, Sugimura T, Matsushima T: Environmental mutagens and carcinogens. Annu Rev Genet 12:117–159, 1978

(*11*) Sugimura T, Sato S, Nagao M, et al: Overlapping of car-

cinogens and mutagens. *In* Fundamentals in Cancer Prevention, Proceedings of the 6th International Symposium of the Princess Takamatsu Cancer Research Fund 1975 (Magee P, Tokayama S, Sugimura T, eds). Tokyo: Univ Tokyo Press, 1976, pp 191–215

(*12*) McCann J, Choi E, Yamasaki E, et al: Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. Proc Natl Acad Sci USA 72:5135–5139, 1975

(*13*) Williams GM: The detection of chemical mutagens/carcinogens by DNA repair and mutagenesis in liver cultures. *In* Chemical Mutagens, vol 6 (DeSerres FJ, Hollaender A, eds). New York: Plenum Press, 1980, pp 61–79

(*14*) Weisburger EK, Weisburger JH: Chemistry, carcinogenicity, and metabolism of 2-fluorenamine and related compounds. Adv Cancer Res 5:333–430, 1958

(*15*) Miller JA, Miller EC: The metabolic activation of carcinogenic aromatic amines and amides. Prog Exp Tumor Res 11:273–301, 1969

(*16*) Weisburger JH, Weisburger EK: Tests for chemical carcinogens. *In* Methods In Cancer Research, vol 1 (Busch H, ed). New York: Academic Press, 1967, pp 307–398

(*17*) Weisburger JH, Williams GM: Chemical carcinogenesis. *In* Toxicology, The Basic Science of Poisons (Doull J, Klaasen C, Amdur M, eds). New York: Macmillan, 1980, pp 84–138

(*18*) Weisburger JH: Bioassays and tests for chemical carcinogens. *In* Chemical Carcinogens, American Chemical Society Monogr No. 173 (Searle CE, ed). Washington, D.C.: Am Chem Soc, 1976, pp 1–23

(*19*) Page NP: Current concepts of a bioassay program in environmental carcinogenesis. *In* Advances in Modern Toxicology, vol 3 (Kraybill HF, Mehlman MA, eds). New York: Wiley, 1977, pp 87–172

(*20*) Kriek E: Aromatic amines and related compounds as carcinogenic hazards to man. *In* Environmental Carcinogenesis. Occurrence, Risk Evaluation and Mechanisms. (Emmelot P, Kriek E, eds). Amsterdam: Elsevier/North Holland, 1979, pp 143–164

(*21*) Weisburger JH, Weisburger EK: Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(*22*) Irving CC: Species and tissue variation in the metabolic activation of aromatic amines. *In* Carcinogen Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 211–228

(*23*) Morris HP: Studies on the development, biochemistry, and biology of experimental hepatomas. Adv Cancer Res 9:228–303, 1965

(*24*) Ito N, Hiasa Y, Konishi Y, et al: The development of carcinoma in liver of rats treated with *m*-toluenediamine and the synergistic and antagonistic effects with other chemicals. Cancer Res 29:1137–1145, 1969

(*25*) Kiese M: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol Rev 18: 1091–1161, 1966

(*26*) Uehleke H: N-Hydroxylation of carcinogenic amines by bladder mucosa. *In* Bladder Cancer, a Symposium (Lampe K, ed). Birmingham, Alabama: Aesculapius, 1967, pp 98–106

(*27*) Bengtsson U, Johansson S, Angervall L: Malignancies of the urinary tract and their relation to analgesic abuse. Kidney Int 13:107–113, 1978

(*28*) Isaka H, Yoshii H, Otsuji A, et al: Tumors of Sprague-Dawley rats induced by long-term feeding of phenacetin. Gan 70:29–36, 1979

(*29*) Vaught JB, King CM: Phenacetin studies. Science 206: 637–639, 1979

(*30*) Walpole AL: Neuere chemische Carcinogene und deren möglicher Wirkungsmechanismus. *In* Berliner Symposium Uber Fragen der Carcinogenese, 1959 (Kraatz H, Graffi A, Gummel H, et al, eds). Berlin: Akademie-Verlag, 1960, pp 9–11

(*31*) Weisburger EK: Laboratory chemicals: *N*-2-fluorenylacetamide and derivatives. *In* Carcinogens in Industry and Environment (Sontag J, ed). New York: Marcel Dekker, 1981, pp 583–666

(*32*) Weisburger JH, Grantham PH, van Horn EC, et al: Activation and detoxification of *N*-2-fluorenylacetamide in man. Cancer Res 24:475–479, 1964

(*33*) Homburger F: Chemical carcinogenesis in Syriam hamsters: A review (through 1976). Prog Exp Tumor Res 23:100–179, 1979

(*34*) Miller EC, Miller JA, Enomoto M: The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamsters, and guinea pigs. Cancer Res 24:2018–2031, 1964

(*35*) Grantham PH, Mohan CC, Weisburger EK, et al: Identification of new water-soluble metabolites of acetanilide. Xenobiotica 4:69–76, 1974

(*36*) Bralow SP, Weisburger JH: Experimental carcinogenesis in the digestive organs. Clin Enterol 5:527–542, 1976

(*37*) Williams GM, Chandrasekaran V, Katayama S, et al: Carcinogenicity of 3-methyl-2-naphthylamine and 3,2′-dimethyl-4-aminodiphenyl to the bladder and gastrointestinal tract of the Syrian golden hamster with atypical proliferation enteritis. JNCI 67:481–488, 1981

(*38*) Spingarn NE, Weisburger JH: Formation of mutagens in cooked foods. I. Beef. Cancer Lett 7:259–264, 1979

(*39*) Kasai H, Nishimura S., Nagao M, et al: Fractionation of a mutagenic principle from broiled fish by high-pressure liquid chromatography. Cancer Lett 7:343–348, 1979

(*40*) Spingarn NE, Garvie CT: Formation of mutagens in sugar-ammonia model systems. J Agric Food Chem 27: 1319–1321, 1979

(*41*) Reddy BS, Cohen LA, McCoy D, et al: Nutrition and its relationship to cancer. Adv Cancer Res 32:237–345, 1980

(*42*) Weisburger JH: Mechanism of action of diet as a carcinogen. Cancer 43:1987–1995, 1979

(*43*) Gelboin HV, Ts'o PO, eds: Polycyclic Hydrocarbons and Cancer, vol 1. New York: Academic Press, 1978

(*44*) Williams JR, Grantham PH, Marsh HH III, et al: The participation of liver fractions and of intestinal bacteria in the metabolism of *N*-hydroxy-*N*-2-fluorenylacetamide in the rat. Biochem Pharmacol 19:173–188, 1970

(*45*) Weisburger EK, Russfield AG, Homburger F, et al: Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. J Environ Pathol Toxicol 2:325–356, 1978

Some Conclusions Derived From a Liver Model for Carcinogenesis[1]

Ross Cameron *and* Emmanuel Farber[2]

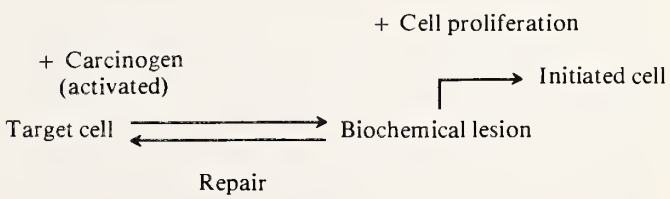
ABSTRACT—A new model of liver cancer development with chemicals is described. This model was based on the hypothesis that chemical carcinogens induced as a first step altered hepatocytes that are resistant to the inhibitory effect of a carcinogen, such as *N*-2-fluorenylacetamide, on cell proliferation. After the administration of a single initiating dose of a carcinogen, the rare resistant hepatocyte is selected by the creation of a special selection pressure, consisting of a stimulus for cell proliferation in the presence of an environment that inhibits normal hepatocyte proliferation. The latter is created by brief exposure to dietary *N*-2-fluorenylacetamide. With this approach, initiated hepatocytes and large hyperplastic liver nodules can be rapidly induced in a synchronized fashion. A direct material continuity between resistant hepatocytes, foci, and nodules of such cells (hyperplastic nodules) and hepatocellular carcinoma was established with diethylnitrosamine as the initiating carcinogen. The use of the resistant cell model has shown that initiation consisted of at least two steps, the second of which is a compulsory round of cell proliferation. With this model, three mechanisms of promotion in the liver are suggested: differential inhibition, differential stimulation, and differential recovery. The relationship of these early changes to liver cancer development is discussed.—Natl Cancer Inst Monogr 58: 49–53, 1981.

NEED FOR ANALYZABLE MODELS IN THE SEQUENTIAL STUDY OF THE CARCINOGENIC PROCESS

The development of cancer in the liver and in other tissues is a multistep process that occurs over many months in animals and years in man (*1–5*). Liver cancer has been induced experimentally with a wide variety of chemicals (*4–6*), radiation, and at least two viruses (*4, 7*). By far, most studies have used chemicals usually in regimens of continuous or discontinuous chronic exposure (*4–6*) and have revealed various cellular and tissue changes which regularly occur prior to the appearance of cancer. Of these changes, four, i.e., hyperplastic areas or islands, early and late hyperplastic nodules, and hyperbasophilic areas (*4, 5*) have been identified as part of a "material continuity" (*1, 3, 5*) that leads to liver cancer. Chronic exposure to chemicals, although often effective in inducing a high incidence of cancer of the liver, leads to a mixture of cellular responses and overlapping sequences. With each exposure to a chemical that may have a major modulating effect at one or more phases of the process, multiple parallel and staggered reactions will result that make any sequential analyses virtually impossible (*5*). In recent years, carcinogenic regimens by which investigators attempt to segregate various phases of the carcinogenic process in the liver have been designed by several groups including Peraino et al. (*8, 9*), Scherer and Emmelot (*10, 11*), Pitot and co-workers (*12*), and those in this laboratory (*5, 7, 13–19*). All these various models have been recently compared and discussed in detail (*5, 7*), so that in this paper only some of the major conclusions and perspectives derived from our model will be presented. Although our model is yet to be exploited fully, it has already been possible to pose serious and important questions, such as the role of cell proliferation in initiation (*5, 16–18*), the induction potential of a variety of carcinogens (*5, 7, 18, 19*), possible mechanisms of promotion, and concepts concerning the nature of the later phases of promotion and cancer development as discussed below.

INITIATION

Initiation of chemical carcinogenesis of the liver has been shown to be a two step process (*16–19*):



A single exposure to a chemical carcinogen such as DEN alone at high doses can ultimately result in liver cancer (*7*). In addition, coupling cell proliferation by either partial hepatectomy or carbon tetrachloride with a single exposure to a number of chemical carcinogens, including DEN, DMBA, DMN, MNU, methylazoxymethanol, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, urethan, and thioacetamide, could induce liver cancer (*20*). The only means by which one can determine that initiation has occurred, however, is the appearance of liver cancer 1 to 2 years after the chemical exposure. The carcinogen-altered or "initiated" cell

Abbreviations: DEN = diethylnitrosamine; DMBA = 7,12-dimethylbenz[*a*]anthracene; DMN = dimethylnitrosamine; MNU = methylnitrosourea; 2-FAA = *N*-2-fluorenylacetamide; BP = benzo[*a*]pyrene; DMH = dimethylhydrazine.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

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populations are not evident, nor do they show independent or autonomous growth (*5*) following exposure to the carcinogen; to assay for initiation, we required some method by which these cells could be made to proliferate selectively. The finding that hepatocytes of both ethionine- and 2-FAA-induced nodules shared the property of a relative resistance to the cytotoxic and "mitoinhibitory" effects of certain hepatocarcinogens and hepatotoxins (*21*) suggested a possible basis for applying a selective proliferation to initiated hepatocytes. If such carcinogen-altered cells showed resistance and could be stimulated to grow while at the same time the surrounding noninitiated cells are inhibited in their growth, then a selective amplification of the initiated resistant population could be achieved (*13, 14*).

In the original design, the initiating carcinogen was DEN given at a single necrogenic (*see* below) dose of 200 mg/kg to adult male rats. Two weeks later, the animals were exposed for 2 weeks to 2-FAA, a potent mitoinhibitor for normal hepatocyte proliferation, and during that same period they had a 70% partial hepatectomy (*13, 14*). The DEN-altered hepatocytes, resistant to the mitoinhibitory effects of the 2-FAA, proliferated immediately after the partial hepatectomy and grew rapidly so that in a week they were visible grossly as hyperplastic foci, scattered randomly throughout the liver. Most (90–95%) such foci stained positive histochemically for the enzyme γ-glutamyl transferase (*15, 22, 23*), which then served as a convenient marker for quantitation of foci. The number of enzyme-positive foci selectable with 2-FAA plus the partial hepatectomy varies linearly with the dose of DEN in a necrogenic dose range of 25–200 mg/kg (*13, 14*). An important aspect crucial to the use of this model is whether the resistant cells, as a population, are the precursors for liver cancer; this has been demonstrated unequivocally for DEN (*14*). Thus we feel justified in designating the induction of such resistant hepatocytes as "initiation." Also, efforts are being made to find a substitute for the dietary 2-FAA in the selection procedure. Recent results from the laboratory of Lombardi and Shinozuka suggest that a choline-deficient diet may be effective (*24*). Whether the same resistant population is being selected by choline deficiency and by 2-FAA plus a partial hepatectomy remains to be established.

An important question addressed by this model is the role of cell proliferation in initiation. The finding that a number of chemicals, including α-hexachlorocyclohexane, thioacetamide, and carbon tetrachloride, could serve as effective alternatives to partial hepatectomy as the proliferative stimulus in this selection phase (*25*) allowed the surgical procedure to be incorporated into the initiation phase (*16, 17*). Cayama (*16*) found that certain carcinogens, e.g., MNU, which when given alone did not induce selectable foci, could initiate if the exposure to the MNU was coupled with a partial hepatectomy. When several carcinogens were used as initiators, i.e., MNU, DEN, DMN, BP, or DMH, the surgery had to be performed within a few days of exposure to the chemical for the chemical to be effective in initiation (*16, 17, 26*). The liver appears to be most sensitive to initiation if a direct-acting carcinogen such as MNU is given between 18 and 24 hours after the partial hepatectomy (*16*). With indirect-acting carcinogens, such as BP (*19*) or DMH (*26*), 6 to 18 hours is the period of maximum susceptibility. The ability of carcinogens such as DEN to initiate at necrogenic doses was related primarily to the compensatory cell proliferation accompanying the necrosis in the following manner. The necrogenic effects of DEN were inhibited by diethyldithiocarbamate administered several hours after the DEN, which markedly decreased the degree of initiation. However, it could be restored if the surgery was also performed within a few days of administration of the DEN (*17*). Therefore, cell proliferation must occur within a brief period after the exposure to a chemical carcinogen for development of neoplasia.

Various chemical carcinogens of diverse action and structure were tested for their ability to induce 2-FAA-resistant hepatocytes that could be selected and quantified. Thirty chemicals given as a single dose within 12 hours after partial hepatectomy, including 5 polycyclic aromatic hydrocarbons, various nitroso compounds, aromatic amines, and others (*18*) induced 10 to 45 γ-glutamyl transferase-positive foci/cm² in the liver (*18, 19*). With 4 of these chemicals as initiators, i.e., DEN, BP, DMBA, and MNU, liver cancers appeared by 8 to 12 months after selection, so that the model for induction of foci appears to be directly relevant also to the induction of liver cancer. The consistency with which the known chemical carcinogens tested thus far can induce these resistant foci suggests that this approach may serve as the basis for a short-term in vivo bioassay for the initiating ability of various chemicals and xenobiotics (*18, 19*). The ability to induce nodules and liver cancer with chemicals of such diverse nature as DEN, DMH, MNU, or BP will be valuable in the assessment of molecular and cellular changes related to the exposure to a specific chemical and those related to the carcinogenic process.

Another important characteristic of initiation is its apparent irreversibility. The 2-FAA-resistant hepatocytes induced by a single dose of DEN were selectable with 2-FAA plus a partial hepatectomy up to 36 weeks after initiation with DEN (*27*). A similar persistence of these resistant hepatocytes in the livers of animals given MNU and surgical treatment was observed (*27*). Therefore, it appears that once resistant cells are induced by exposure to a carcinogen coupled with cell proliferation, such cells are not repaired or removed for many months, if at all (*5*).

RESISTANT HEPATOCYTES AS ONE POPULATION OF INITIATED CELLS

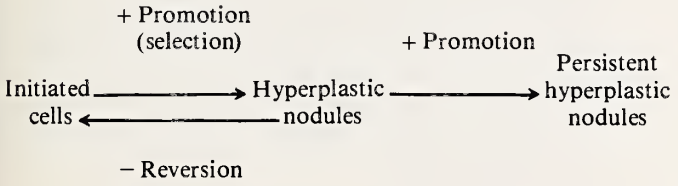
The resistant hepatocytes that are induced by various carcinogens (*7, 18, 19*) and can be made to proliferate selectively into hyperplastic nodules are part of a material continuity that eventuates in liver cancer. These resistant hepatocytes as measured by the number of foci selectable with 2-FAA plus partial hepatectomy serve as one means of assaying initiation quantitatively (*5*). The number of 2-FAA-resistant foci is proportional to the dose of initiating carcinogen as determined for DEN (*13*), BP (*18, 19*), and DMH (*26*). Assuming an origin of each focus from a single cell, we estimate that 1 resistant hepatocyte is in-

duced per 10^6 original hepatocytes with the maximum dose of DEN or BP or about 10^3 resistant hepatocytes (and foci) per 5 g liver. Initiation as assayed by induction of resistant cell populations is clearly a rare event (*5, 15*) with a frequency ($1/10^6$) approaching that of a mutation.

This property of resistance to the cytotoxic effects of a hepatocarcinogen, i.e., 2-FAA, shown by the early carcinogen-altered hepatocytes is also manifested by the hepatocytes of hyperplastic nodules which in addition are resistant to the necrogenic effects of carbon tetrachloride or DMN in vivo (*21*). Some possible bases for such resistance of hyperplastic nodules may relate to the 80% inhibition of uptake for 2-FAA (*21*) or to the changes in microsomal enzymes responsible for activation and inactivation of carcinogens, i.e., a large decrease in the concentration of cytochrome P_{450} and of aryl hydrocarbon hydroxylase (*28*) and a marked increase in the activity of epoxide hydratase (*29, 30*) accompanied by the induction of a novel hemopolypeptide (*31*).

PROMOTION

Promotion in the liver in this model appears to consist of the differential stimulation by selection of resistant initiated hepatocytes to form nodules that persist for several months (*5, 7, 15*).



The process whereby initiated cells are encouraged or stimulated to evolve into cancer is called "promotion" and agents that favor or accelerate the process are called "promoters" (*1, 3, 5*). In the liver, a focal proliferation of hepatocytes could be the result of at least three mechanisms: a) differential inhibition, b) differential stimulation, or c) differential recovery.

Differential Inhibition

This is the basis for selection in the resistant cell model by 2-FAA, which on the one hand inhibits normal hepatocyte proliferation but at the same time allows the rapid growth of 2-FAA-resistant hepatocytes when a stimulus for proliferation is applied (*13*). Recent unpublished experiments (Stora C: Personal communication) which show fluorescence of 2-FAA in the hepatocytes surrounding hyperplastic nodules, but not within the nodule cells of animals fed 2-FAA, provide further support for differential inhibition by a hepatocarcinogen as a possible mechanism of promotion.

Differential Stimulation

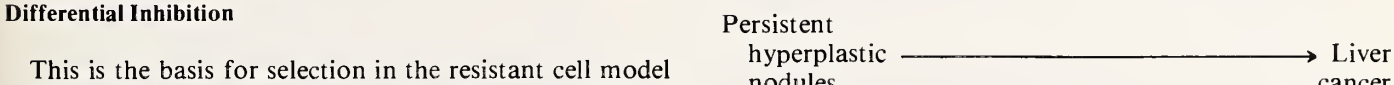
An initiated cell can grow and develop into a nodule if a promoter selectively stimulates that cell to grow. Several agents that are liver mitogens and microsomal enzyme inducers, e.g., α-hexachlorocyclohexane and cyproterone acetate have increased dramatically the ^{3}H-index of DEN-initiated islands or foci to as much as 40% compared with 5% for the surrounding hepatocytes after treatment with cyproterone acetate (*32*). This differential effect is accentuated with the increasing age of rats as the responsiveness of the surrounding hepatocytes to these agents diminishes and the stimulatory effect on the preneoplastic cells is maintained (*32*). In this way, a differential stimulation is created that generates a progressive enlargement of focal areas of hyperplasia.

Differential Recovery

Most focal hyperplastic lesions such as hyperplastic nodules undergo reversion ("remodeling" or "maturation") to normal-appearing liver as measured morphologically and histochemically (*4, 5, 14, 23*). The rate of reversion varies between two extremes with some nodules showing rapid reversion and others showing none (persistent hyperplastic nodules). This process of reversion may be similar in principle to the return of the liver to a resting phase (G_0?) after one or more cycles of cell proliferation. However, the nuclear structure of a nodule hepatocyte with its characteristic loose open chromatin pattern and large nucleoli (*4, 14*) does not revert to that of a resting hepatocyte but resembles that of a proliferating cell, even after cell proliferation is essentially complete. This type of differential recovery or reversion in effect may lead to the promotion of some preneoplastic hepatocytes.

CANCER DEVELOPMENT

The first neoplastic cells arise in many instances from within the confines of persistent hyperplastic nodules (*4, 5, 14*), but the number of cancers is much less than the number of nodule cells that can serve as precursors and have been estimated to be about 1 cancer cell per 10^6 nodule cells: an event of equally rare occurrence as the initiation event [termed the "2nd rare event"; (*5, 7*)].



The precise nature of this event or even the number of steps involved in the nodule to cancer sequence is not known. Autonomous growth was not found when nodules were transplanted into mammary fat pads, i.e., sites where hepatomas grew readily (*33*). Also, no growth of hyperplastic nodule tissue was observed when injected into various sites, including the liver of syngeneic rats or of irradiated animals given cortisone (*34*). The understanding of the nodule to cancer sequence will require the development of appropriate analyzable models which control and synchronize each step as is now being developed for the analysis of the early steps.

PERSPECTIVES

The model for the sequential analysis of development of liver cancer has allowed significant progress in the delineation of the first few steps and the beginning of a framework by which further steps can be understood. An extensive analysis of various experimental systems and human examples of carcinogenesis in which a sequence could be determined has recently been completed (*5*) and reveals that many of the generalities derived from the liver model concerning initiation, promotion, and cancer development are strikingly similar to conclusions derived from other systems such as the skin and urinary bladder. However, the liver is the first system in which a testable hypothesis concerning the nature of the first carcinogen-induced cell, i.e., the resistant cell and how it might relate to the carcinogenic process has been proposed and subjected to experimental analysis. The fact that an environment can be created in vivo that allows the testing of hypotheses should encourage the development of alternative ones and allow the beginning of the analysis of the principles of promotion and of cancer development.

REFERENCES

(*1*) FOULDS L: Neoplastic Development, vol 1. New York: Academic Press, 1969

(*2*) FARBER E: Carcinogenesis-cellular evolution as a unifying thread. Presidential address. Cancer Res 33:2537-2550, 1973

(*3*) FOULDS L: Neoplastic Development, vol 2. New York: Academic Press, 1975

(*4*) FARBER E: The pathology of experimental liver cancer. *In* Liver Cell Cancer (Cameron HM, Linsell DA, Warwick GP, eds). Amsterdam: Elsevier/North Holland, 1976, pp 243-277

(*5*) FARBER E, CAMERON R: The sequential analysis of cancer development. Adv Cancer Res 31:125-226, 1980

(*6*) WOGAN GM: The induction of liver cell cancer by chemicals. *In* Liver Cell Cancer (Cameron HM, Linsell DA, Warwick GP, eds). Amsterdam: Elsevier/North Holland, 1976, pp 121-152

(*7*) FARBER E: Response of liver to carcinogens—a new analytical approach. *In* Toxic Liver Injury (Farber E, Fischer MM, eds). New York: Marcel Dekker, 1979, pp 445-467

(*8*) PERAINO C, FRY RJ, STAFFELDT E: Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res 31:1506-1512, 1971

(*9*) PERAINO C, FRY RJ, STAFFELDT E, et al: Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenylchloroethane on 2-AAF-induced hepatic tumorigenesis in the rat. Cancer Res 35:2884-2890, 1975

(*10*) SCHERER E, EMMELOT P: Foci of altered liver cells induced by a single dose of diethylnitrosamine and partial hepatectomy: Their contribution to hepatocarcinogenesis in the rat. Eur J Cancer 11:145-154, 1975

(*11*) _____: Kinetics of induction and growth of enzyme-deficient islands involved in hepatocarcinogenesis. Cancer Res 36:2544-2554, 1976

(*12*) PITOT HC, BARSNESS L, GOLDSWORTHY T, et al: Biochemical characterization of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. Nature 271:456-458, 1978

(*13*) SOLT DB, FARBER E: New principle for the analysis of chemical carcinogenesis. Nature 263:701-703, 1976

(*14*) SOLT DB, MEDLINE A, FARBER E: Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am J Pathol 88:595-618, 1977

(*15*) FARBER E, CAMERON RG, LAISHES B, et al: Physiological and molecular markers during carcinogenesis. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Show CR, eds). New York: Raven Press, 1979, pp 319-335

(*16*) CAYAMA E, TSUDA H, SARMA DS, et al: Initiation of chemical carcinogenesis requires cell proliferation. Nature 275:60-62, 1978

(*17*) YING TS, SARMA DS: Role of liver cell necrosis in the induction of preneoplastic lesions. Proc Am Assoc Cancer Res 20:56, 1979

(*18*) TSUDA H, LEE, G, FARBER E: Induction of resistant hepatocytes as a new principle for a possible short-term in vivo test for carcinogens. Cancer Res 40:1157-1164, 1980

(*19*) _____: Resistant hepatocytes as early changes in liver induced by polycyclic aromatic hydrocarbons. Int J Cancer 25: 137-139, 1980

(*20*) CRADDOCK VM: Cell proliferation and experimental liver cancer. *In* Liver Cell Cancer (Cameron HM, Linsell DA, Warwick GP, eds). Amsterdam: Elsevier/North Holland, 1976, pp 153-201

(*21*) FARBER E, PARKER S, GRUENSTEIN M: The resistance of putative premalignant liver cell populations, hyperplastic nodules, to the acute cytotoxic effects of some hepatocarcinogens. Cancer Res 36:3879-3887, 1976

(*22*) CAMERON R, KELLEN J, KOLIN A, et al: Gamma-glutamyltransferase in putative premalignant liver cell populations during hepatocarcinogenesis. Cancer Res 38: 823-829, 1978

(*23*) OGAWA K, SOLT DB, FARBER E: Phenotypic diversity as an early property of putative preneoplastic hepatocyte populations in liver carcinogenesis. Cancer Res 40:725-733, 1980

(*24*) SELLS MA, KATYAL SL, SELL S, et al: Induction of foci and altered γ-glutamyltranspeptidase-positive hepatocytes in carcinogen-treated rats fed a choline-deficient diet. Br J Cancer 40:274-283, 1979

(*25*) CAMERON R, LEE G, FARBER E: Chemical mitogens as effective alternatives to partial hepatectomy in a new model for the sequential analysis of hepatocarcinogenesis. Proc Am Assoc Cancer Res 19: 222, 1978

(*26*) YING TS, SARMA DS, FARBER E: Induction of presumptive preneoplastic lesions in rat liver by a single dose of 1,2-dimethylhydrazine. Chem Biol Interact 28:363-366, 1979

(*27*) SOLT D, CAYAMA E, SARMA DS, et al: Persistence of resistant putative preneoplastic hepatocytes induced by *N*-nitrosodiethylamine or *N*-methyl-*N*-nitrosourea. Cancer Res 40:1112-1118, 1980

(*28*) CAMERON R, SWEENEY GD, JONES K, et al: A relative deficiency of cytochrome P_{450} and aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase in hyperplastic nodules induced by 2-acetylaminofluorene in rat liver. Cancer Res 36:3888-3893, 1976

(*29*) LEVIN W, LU AY, THOMAS PE, et al: Identification of epoxide hydrase as the preneoplastic antigen in rat liver hyperplastic nodules. Proc Natl Acad Sci USA 75:3240-3243, 1978

(*30*) Sharma RN, Gurtoo HL, Farber E, et al: Effects of hepatocarcinogens and hepatocarcinogenesis on the activity of rat liver microsomal epoxide hydrolase and observations on the electrophoretic behavior of this enzyme. Cancer Res 41:3311–3319, 1981

(*31*) Cameron R, Murray RK, Farber E, et al: Some patterns of response of liver to environmental agents. Ann NY Acad Sci 329:39–47, 1979

(*32*) Ohde G, Schuppler J, Schulte-Hermann R, et al: Proliferation of rat liver cells in preneoplastic nodules after stimulation of liver growth by xenobiotic inducers. Arch Toxicol 2 (Suppl): 451–455, 1979

(*33*) Williams GM, Klaiber M, Farber E: Differences in growth of transplants of liver, liver hyperplastic nodules and hepatocellular carcinomas in the mammary fat pad. Am J Pathol 89:379–390, 1977

(*34*) Farber E: Hyperplastic liver nodules. Methods Cancer Res 7:345–375, 1973

Initiation and Promotion of Liver Tumorigenesis[1, 2]

Carl Peraino[3]

ABSTRACT—Characteristics of the initiation and promotion stages of hepatic tumorigenesis are derived from published data and from the results of new experiments. The general experimental protocol in all studies has involved the short-term feeding of a diet containing the hepatocarcinogen *N*-2-fluorenylacetamide (2-FAA) as the initiating stimulus followed by the feeding of a diet containing phenobarbital as the promoting stimulus. Modifications of this sequential treatment protocol have included changes in the: 1) duration of 2-FAA feeding, 2) duration of phenobarbital feeding, 3) interval between the termination of feeding the first chemical and the onset of the second, and 4) dietary concentration of phenobarbital. On the basis of these experiments, the following conclusions have been drawn: 1) The predominant effect of brief exposure to 2-FAA is tumor initiation; the promoting activity of 2-FAA becomes apparent as the duration of its administration is increased. 2) The hepatocytes initiated by 2-FAA persist and are responsive to the promoting action of phenobarbital long after the carcinogen treatment is discontinued. 3) The promoting effects of phenobarbital in 2-FAA-initiated hepatocytes may be irreversible. 4) Promotion by phenobarbital involves primarily an increase in the probability that initiated hepatocytes will express the neoplastic phenotype and does not affect the time course of this expression or the characteristics (growth rate and degree of differentiation) of the tumors that ultimately appear. A useful strategy for the identification of the critical molecular events in tumor promotion might involve cross-model comparisons of biochemical responses to promoters in two verified initiation–promotion models, such as skin and liver, that are amenable to experimental manipulation. Promoter effects common to both models would be classified as potentially relevant to the promotion mechanism, whereas those effects that are model specific could be eliminated from further consideration.—Natl Cancer Inst Monogr 58: 55–61, 1981.

Experimental evidence for the existence of stages in the onset of neoplasia was obtained more than 50 years ago in studies showing that the physical wounding of skin previously treated with coal tar resulted in skin tumor formation (*1*). Subsequent experiments, in which physical trauma was replaced by the application of chemical irritants, yielded similar results (*2, 3*). These observations were codified in 1944 by Rous, who coined the now classic terms "initiation" and "promotion" to denote, respectively, 1) the production of potentially tumorigenic cells by limited carcinogen treatment, and 2) the completion of the neoplastic transformation as the result of subsequent therapy with any of a wide variety of noncarcinogenic irritants and proliferative stimuli (*3*).

Major additional advances in the study of multistage skin tumorigenesis included the discovery of the potent tumor-promoting activity of croton oil (*4*) and the observation that the initiation stage is irreversible, whereas the promotion stage is reversible (*5*). The subsequent identification of tetradecanoyl phorbol acetate as the active ingredient of croton oil (*6*) has led to the use of this and other phorbol esters in a wide variety of investigations aimed at elucidation of the mechanism of tumor promotion (*7–11*). Such studies have disclosed that phorbol esters active as promoters exert a broad range of biologic and biochemical effects in vivo and in vitro. However, no one has yet demonstrated a causal link between any of these changes and tumor promotion. The difficulty in identifying such a relationship stems primarily from the paucity of well-defined initiation–promotion models of tumorigenesis amenable to comparative biologic and biochemical analysis. The existence of different models that can be judiciously compared would permit the identification of those biochemical characteristics common to the various models and hence potentially relevant to the tumorigenic process.

Our efforts over the past 10 years have been directed toward the development of the liver as an initiation–promotion model that can be exploited at least as intensively as has the skin system, thereby providing a basis for cross-model comparisons, as suggested above. The following discussion summarizes what is presently known about the characteristics of the different stages of tumorigenesis in liver.

DEMONSTRATION OF INITIATION AND PROMOTION IN LIVER TUMORIGENESIS

Indirect evidence that liver tumorigenesis occurs in stages was obtained several years ago in numerous studies. Cole and Nowell (*12*) observed that the administration of the hepatotoxin, carbon tetrachloride, to mice that had been previously X-irradiated substantially increased tumor incidence over that in mice receiving only the irradiation. Examination by Farber (*13, 14*) of the various types of nodular hepatic lesions occurring during prolonged carcinogen treatment has led to the suggestion that malignant

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; DMN = dimethylnitrosamine; DMBA = 7,12-dimethylbenz[*a*]anthracene.

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liver tumors evolve from cells contained in certain of the characteristic lesions, termed "hyperplastic nodules," that precede tumor formation under these conditions. Further evidence that hyperplastic nodules are tumor precursors was obtained by Teebor and Becker (*15*), who observed that the feeding of 2-FAA to rats for 3 intervals of 3 weeks each, alternating with 1-week intervals on a control diet, produced a high yield of hyperplastic nodules that regressed with a low subsequent incidence of hepatic tumors. However, an additional 3 weeks of treatment with 2-FAA yielded nodules that persisted after the carcinogen was withdrawn as well as a high incidence of hepatic tumors. A later variant of this approach involved the administration of a single dose of DMN following the 3 intervals of 2-FAA ingestion. This combination produced a high tumor yield, though no tumors were generated by either treatment alone (*16*).

The studies cited above indicated that limited carcinogen treatment produces preneoplastic hepatocytes which require subsequent additional stimuli to complete the transformation into frank tumor cells. However, these investigations suffered from a common methodological deficiency, i.e., the use of substances with carcinogenic and/or mutagenic activity as the additional stimuli. Under such conditions, these investigators could not determine whether progressive stages of liver tumorigenesis are qualitatively similar (involving, e.g., the successive accumulation of mutations) or dissimilar, as had been demonstrated for skin tumorigenesis (*7*).

The first definitive evidence that the onset of liver neoplasia proceeds in qualitatively distinct sequential stages emerged from an investigation of the effect of phenobarbital on 2-FAA-induced hepatocarcinogenesis (*17*). This study consisted of a comparison of the effects of two types of 2-FAA–phenobarbital exposure modalities on tumor incidence. In the first instance, 2-FAA and phenobarbital were present in the diet concurrently (simultaneous treatment protocol). Under these conditions, hepatic tumor incidence was substantially less than that produced by feeding the 2-FAA alone. A protective effect of phenobarbital was also noted with the hepatocarcinogens, 4-dimethylaminoazobenzene (*18*) and diethylnitrosamine (*19*), when the simultaneous treatment protocol was used. Inasmuch as phenobarbital is a potent inducer of enzymes that actively metabolize these carcinogens (*20*), one could conclude that the anticarcinogenic action of phenobarbital in these instances stems from a phenobarbital-mediated shift in the balance of carcinogen metabolism toward detoxification and degradation.

The second type of exposure involved the prolonged feeding of phenobarbital after the termination of a brief interval of administration of 2-FAA in the diet (sequential treatment protocol). This regimen produced a markedly greater tumor incidence than that observed in rats receiving only 2-FAA briefly (*17*).

From a consideration of the contrasting effects of the simultaneous and sequential treatment protocols on tumorigenesis it is evident that the enhancing effect of phenobarbital cannot be a consequence of increased metabolic activation of the carcinogen. The hypothesis most consistent with the data is that phenobarbital given according to the sequential treatment protocol facilitates the ultimate expression of tumorigenic changes initiated by prior exposure to the carcinogen. This interpretation implies the existence of at least two elements of the tumorigenic process that differ mechanistically as well as in temporal occurrence and comprises the basis for the argument that the initiation–promotion concept of tumorigenesis applies to the liver.

AGENTS WITH LIVER TUMOR-INITIATING OR -PROMOTING ACTIVITY

Following the initial observation of the promotion of 2-FAA-induced hepatocarcinogenesis by phenobarbital (*17*), several reports appeared which demonstrated that liver tumor initiation and promotion could be produced by a wide variety of agents (table 1) administered sequentially. Among the initiators were one of unknown (and evidently endogenous) origin in C3H mice and two others (2-methyl-*N,N*-dimethyl-4-aminoazobenzene and DMBA) with low intrinsic hepatocarcinogenic activity. The DMBA study indicated that administration of phenobarbital in the sequential treatment protocol can make the liver highly responsive to carcinogens previously considered specific for other tissues. This increased sensitivity raises the possibility that the liver may serve as the basis for the development of a method for assessing tumorigenic potential in a broad spectrum of environmental pollutants.

The promoters of liver tumors shown in table 1, with the possible exception of phorbol, share the capacity to stimulate liver growth (*20*), and certain elements in the array of biochemical events involved in such stimulation may be common to those events involved in the expressions of neoplasia. The characteristics of the putative promoting action of phorbol in liver differ markedly from those of the other promoters shown in table 1; phorbol enhanced DMN-induced hepatic tumorigenesis only when administered to newborn AKR mice, whereas the effects of the other promoters listed were not age dependent. This difference in behavior suggests that the mechanism of the

TABLE 1.—*Liver tumor initiation–promotion studies*

Animal	Initiator	Promoter	References
Rat	2-FAA	Phenobarbital	(*17*)
Mouse	DMN	Phorbol	(*21*)
Mouse	Unknown	Phenobarbital	(*22, 23*)
Rat	3-methyl-*N,N*-dimethyl-4-aminoazobenzene	"	(*24*)
"	Diethylnitrosamine	"	(*25, 26*)
"	2-FAA	DDT	(*27*)
"	Diethylnitrosamine	Polychlorinated biphenyls	(*28*)
"	2-FAA	Butylated hydroxy-toluene	(*29*)
"	*N,N*-dimethyl-4-amino-azobenzene	Phenobarbital	(*30*)
"	2-methyl-*N,N*-dimethyl-4-aminozbobenzene	"	(*31*)
"	DMBA	"	(*32*)

tumor-enhancing activity of phorbol in liver differs from that of the other agents. Of the promoters tested to date, phenobarbital is probably the most suitable for use in mechanistic studies because there is no evidence that this compound produces hepatic or systemic toxicity at dietary concentrations that are highly effective in enhancing hepatic tumorigenesis. The changes that occur in normal liver during chronic phenobarbital treatment are fully reversible (*20*) and appear to represent an adaptive response to the type of functional load imposed on the liver by the continued presence of phenobarbital.

CHARACTERISTICS OF LIVER TUMOR INITIATION AND PROMOTION

An investigation of the characteristics of the different stages of hepatic tumorigenesis, in particular the postinitiation (promotion) phase, has been underway in our laboratory for several years. The following discussion summarizes our published observations and provides a more detailed description of our current studies.

The initial 2-FAA–phenobarbital study examined the effect of altering the duration of the 2-FAA treatment interval before shifting the rats to either a control diet or one containing phenobarbital (*17*). The results showed that increases in the interval of therapy with 2-FAA increased tumor formation in rats that received this carcinogen alone; shifting rats to phenobarbital following each exposure to 2-FAA substantially increased tumor incidence over that in rats shifted to the control diet. The magnitude of the incremental effect of phenobarbital on tumorigenesis increased as the duration of the prior 2-FAA treatment was reduced, which suggested the predominant effect of brief exposure to the carcinogen is the initiation of tumorigenesis. As the treatment with 2-FAA was extended, the promoting activity of the carcinogen emerged, with a consequent reduction in the requirement for an exogenous promoter (phenobarbital). This relationship between the initiating and promoting activities of the hepatocarcinogen resembles the initiating–promoting behavior of skin carcinogens (*7*).

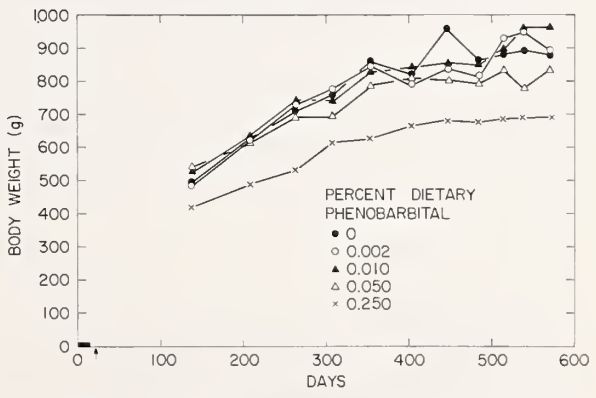
In subsequent investigations (*33, 34*), we examined the effects of varying the interval between the termination of the treatment with 2-FAA and the onset of phenobarbital feeding and the duration of phenobarbital feeding, begun immediately after ending the exposure to the hepatocarcinogen. We observed that the promoting effect of phenobarbital occurred despite the interposition of a 120-day interval between the 2 treatments (*34*). These findings indicate that 2-FAA-modified hepatocytes with tumorigenic potential (i.e., "initiated" hepatocytes) persist long after treatment with 2-FAA has ceased. Such persistence is in accord with evidence obtained from studies on skin tumorigenesis that indicated the initiation stage of tumorigenesis is irreversible (*5, 7, 35*).

Extension of the phenobarbital treatment after exposure to 2-FAA was accompanied by parallel increases in tumor yield (*33, 34*); prolonged exposure to phenobarbital was required for maximal promoting effectiveness (*34*). However, brief (20-day) phenobarbital feeding produced a detectable, though slight, enhancement of tumorigenesis that emerged after the cessation of treatment with the barbiturate (*34*). These results indicated that even a limited exposure of initiated hepatocytes to phenobarbital can irreversibly evoke the expression of carcinogen-induced molecular changes that lead ultimately to neoplasia; prolonging the phenobarbital treatment evidently increases the probability that such expression will occur.

Our most recent study of the characteristics of liver tumor promotion compared the effects of different dietary concentrations of phenobarbital on tumorigenesis in rats previously given 2-FAA (*36*). The experimental procedure involved the feeding of male weanling rats according to the following sequence: 1) 0.02% 2-FAA diet for 14 days; 2) control diet (no additives) for 7 days; 3) diets containing phenobarbital at concentrations of 0% (control), 0.002%, 0.01%, 0.05%, or 0.25% for the remainder of the experiment. At each of the intervals shown in text-figures 1–3, 12 to 24 rats were killed, weighed, and examined for the incidence of liver tumors as described in (*17, 27, 34*).

Text-figure 1 shows the effects of the various phenobarbital treatments on body weight gain throughout the experiment. Apparently in a dietary concentration up to 0.05%, phenobarbital had virtually no intrinsic effect on the growth of the rats; the slightly lower body weights of the rats receiving 0.05% probably resulted from the fact that they had a much higher incidence of hepatic tumors than the controls or those given lower phenobarbital dosages (*see* below). When the concentration of dietary phenobarbital was raised to 0.25%, however, a substantial growth suppression occurred that was evident even at a time when tumor incidence in this treatment group was less than 10% (*see* below). In additional experiments, we observed that the growth suppression produced by 0.25% dietary phenobarbital resulted from a phenobarbital-mediated loss in the efficiency of food utilization for growth (grams body weight gained per gram of diet consumed) rather than a reduction in food intake.These studies will be reported in detail elsewhere.

Rats not given phenobarbital following the initiating 2-FAA treatment showed no tumors until more than 300 days elapsed; percentage tumor incidence then rose to a

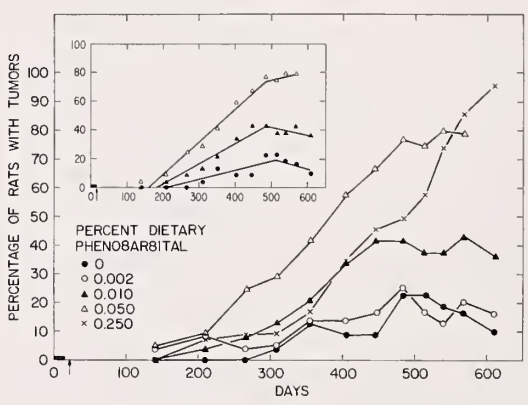


TEXT-FIGURE 1.—Body weight gain in rats (12–24 rats/experimental point) chronically fed different dietary concentrations of phenobarbital beginning (*arrow* on abscissa) 7 days after a 14-day feeding (*black bar* on abscissa) of 2-FAA at a concentration of 0.02%. Text-figure is reproduced from (*36*) with permission of the publisher.

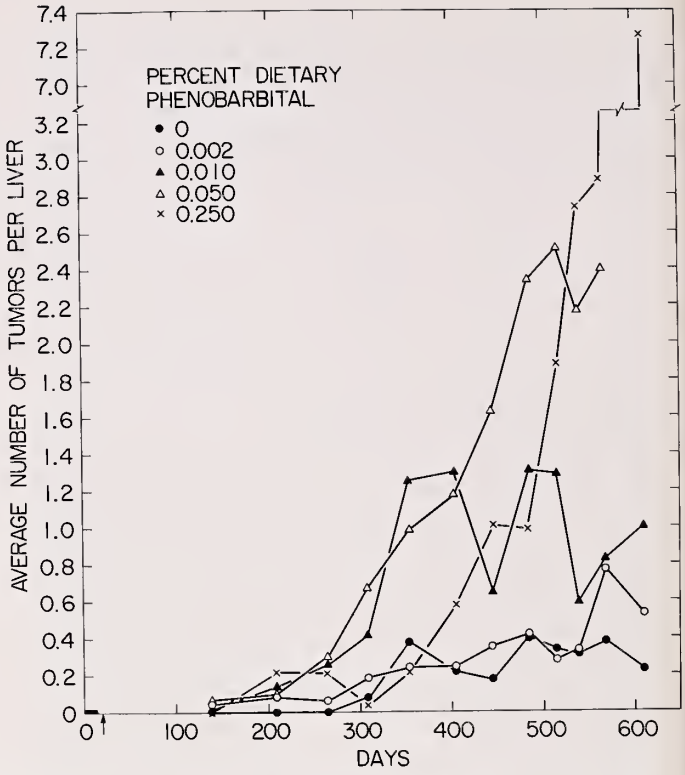
maximum of 20% by 475 days and remained constant through the end of the experiment (610 days). Dietary phenobarbital at a concentration of 0.002% had virtually no effect on the percentage of rats with tumors (text-fig. 2) but caused a slight increase in the average number of tumors per liver at the end of the experiment (text-fig. 3). Raising the dietary phenobarbital concentration to 0.01% had a definite enhancing effect on tumorigenesis, with the attainment of maximal tumor incidences approximately double those in the control animals (text-figs. 2, 3). A critical feature of the kinetics of this enhancement is the clear-cut plateau in tumor incidence that occurred despite the continued administration of phenobarbital. This plateau was evident with respect to both the percentage of rats bearing tumors (text-fig. 2) and the average number of tumors per liver (text-fig. 3) When the dietary phenobarbital concentration was raised to 0.05%, tumor incidence increased further and reached a plateau at approximately four times the incidence levels attained on the control diet.

At the highest dietary concentration of phenobarbital (0.25%), the appearance of tumors was considerably delayed in comparison to that in rats receiving 0.05%. Subsequently, however, a substantial increase in tumorigenesis occurred in rats given 0.25% that led to a nearly 100% tumor incidence by the end of the experiment (text-fig. 2) and a high tumor frequency with no indication of the appearance of a plateau (text-fig. 3). Histologic examination of all tumors showed that the marked enhancement of hepatic tumorigenesis engendered by phenobarbital treatment was not accompanied by any increase in tumor malignancy, irrespective of the phenobarbital dosage; most tumors in all treatment groups were highly differentiated trabecular carcinomas.

The insert in text-figure 2 depicts a computer-generated least squares plot of the percentage tumor incidence data from the 0%, 0.01%, and 0.05% phenobarbital treatment groups. Such a plot permits a more rigorous comparison of tumor incidence kinetics at different phenobarbital dosage levels, but it omits the groups that received the lowest and highest dosages. Omission of the lowest group was based on the absence of a significant tumorigenic response to this treatment. The highest phenobarbital group was omitted



TEXT-FIGURE 2.—Tumor incidence (percentage) in the rats described in text-figure 1. Text-figure is reproduced from (*36*) with permission of the publisher.

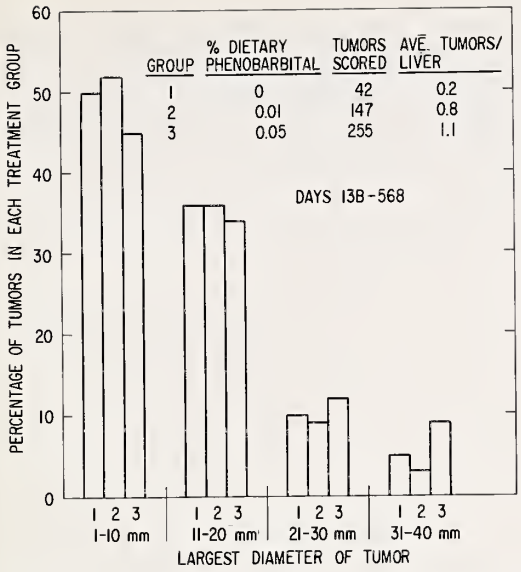


TEXT-FIGURE 3.—Tumor frequency (average No. of tumors/liver) in the rats described in text-figure 1. Text-figure is reproduced from (*36*) with permission of the publisher.

because it is apparent from the growth curves in text-figure 1 and measurements of feed efficiency (*see* text below) that high intakes of dietary phenobarbital cause growth alterations and other metabolic changes not apparent at the lower dosages. Such changes can influence tumorigenesis by mechanisms other than those related directly to the promoting action of phenobarbital (*37, 38*) and might therefore complicate the interpretation of the effects of the barbituate on tumor incidence kinetics. The data in the insert show that within the specified phenobarbital dose range neither the time at which 2-FAA-induced tumors began to appear nor the time at which tumorigenesis reached a plateau phase was influenced by the presence of dietary phenobarbital, although the final tumor incidence level was clearly dependent on its concentration.

The tumor size distribution patterns of the 3 treatment groups shown in the insert in text-figure 2 are given in text figure 4. We separated the tumors into four classes by size and compared the treatment groups with respect to the percentages of the total number of tumors in each group that fell into each size class during the indicated experimental interval (138–568 days after the start of the 14-day 2-FAA treatment). These data show that the enhancement of tumorigenesis by dietary phenobarbital was not accompanied by changes in the distribution of tumor sizes. Therefore, phenobarbital did not influence the growth rates of these tumors.

Collectively, the results of our current investigation clearly indicate that hepatic tumor promotion by pheno-



TEXT-FIGURE 4—Tumor size distributions in those rats in text-figure 1 that received 2-FAA alone or followed by 0.01 or 0.05% dietary phenobarbital. Tabulated data indicating average No. of tumors/liver were calculated over entire experimental interval monitored (158–568 days, as designated). Text-figure is reproduced from (*36*) with permission of the publisher.

barbital has sharply circumscribed mechanistic boundaries. Thus the sole manifestation of its promoting action is an increase in the probability that initiated hepatocytes will express their neoplastic character, as demonstrated by the dose-dependent enhancing effect of phenobarbital on plateau levels of tumor incidence in rats treated with 2-FAA. Other elements of hepatic neoplasia, i.e., the interval before the first appearance of tumors, tumor growth rates, and the degree of tumor differentiation, are unaffected by phenobarbital. In the 3 groups depicted in the text-figure 2 insert, each treatment exerted its full effect, but the tumor incidence in each case was not the maximum attainable. This type of graded response suggests that 1) the 2-FAA treatment alone generated a population of initiated cells incapable of expressing the transformed phenotype without additional stimulation by a promoter and 2) a given dosage of phenobarbital completes the tumorigenic process in a fraction of 2-FAA-initiated cells but is without effect on the tumorigenic potential of the remaining initiated cell population, although these cells are fully capable of expressing such potential at the appropriate phenobarbital dosage.

The inability of phenobarbital (present study) and established skin tumor promoters (*39*) to influence those aspects of the tumorigenic process (i.e., time to first tumor appearance and degree of tumor differentiation) that *are* responsive to carcinogen action (*15, 16, 39, 40*) constitutes strong evidence that phenobarbital is a true promoter with no intrinsic hepatocarcinogenic activity. Additional evidence in support of this conclusion is provided by the occurrence of plateau phases in tumor incidence despite the continued administration of phenobarbital (text-figs. 2, 3). In view of these findings, it is likely that the recently observed incidence of hepatic tumors in Wistar rats fed phenobarbital (*41*) represents the promotion of tumorigenesis initiated either by inadvertent prior exposure to an exogenous carginogen of unknown origin or by the action of an endogenous initiating factor. Support for the latter possibility is provided by prior studies which show that phenobarbital enhanced tumor formation in mice subject to spontaneous hepatic tumorigenesis (*22, 23*) but produced no tumors in mice without this susceptibility (*19*). At present, the mechanism by which phenobarbital furthers hepatic tumorigenesis is unknown, but, given the evidence that promoting dosages of phenobarbital are nonhepatotoxic (*20, 30*), the promoting action of phenobarbital apparently involves biochemical responses well within the physiologic limits for normal hepatocellular metabolism.

The similarities in the characteristics of initiation and promotion in the skin and liver imply that the mechanisms governing the occurrence of these stages are alike in other tissues as well. Thus the rigorous examination of such mechanisms in models amenable to experimental manipulation should provide information relevant to multistage tumorigenesis in general, i.e., irrespective of the tissue in which the process is observed (*8*). On this basis, a valid experimental approach to the analysis of tumor promotion mechanisms might involve, as suggested earlier, cross-model comparisons of biochemical effects of skin and liver tumor promoters in their respective experimental models. Promoter effects common to both models would be classified as potentially relevant to the promotion mechanism, whereas those that are model specific could be eliminated from further consideration.

In view of the evidence cited above for the promoting action of phenobarbital in rodents and the wide-spread human exposure to barbiturates, the possibility that such exposure represents a significant health hazard should be examined. In this regard, recent epidemiologic studies suggest that chronic barbiturate treatment may increase the risk of brain tumorigenesis in children (*42*). Should this effect, currently the subject of considerable dispute (*43–45*), be verified conclusively, it will then be essential that scientists determine whether the barbiturate is acting as a carcinogen or as a promoter under these circumstances. Such information would have important mechanistic and therapeutic implications and would also provide the basis for a rational assessment of the level of human tumorigenesis that results from barbiturate therapy.

REFERENCES

(*1*) DEELMAN HT: The part played by injury and repair in the development of cancer. Br Med J 1:872, 1927

(*2*) ROUS P, KIDD JG: Conditional neoplasms and subthreshold neoplastic states. A study of tar tumors in rabbits. J Exp Med 73:356–389, 1941

(*3*) FRIEDEWALD WF, ROUS P: The initiating and promotion elements in tumor production. J Exp Med 80:101–125, 1944

(*4*) BERENBLUM I: The cocarcinogenic action of croton resin. Cancer Res 1:41–48, 1941

(5) Berenblum I, Shubik P: The persistence of latent tumour cells induced in the mouse's skin by a single application of 9,10-dimethyl-1-1,2-benzanthracene. Br J Cancer 3:384-386, 1949

(6) Hecker E: Phorbol esters from croton oil. Chemical nature and biological activities. Naturwissenschaften 54:282-284, 1967

(7) Salga TJ, Sivak A, Boutwell RK, eds: Carcinogenesis: A comprehensive survey. *In* Mechanisms of Tumor Promotion and Cocarcinogenesis. New York: Raven Press, 1978, pp 11-401

(8) Sivak A: Cocarcinogensis. Biochim Biophys Acta 560:67-89, 1979

(9) Diamond L, O'Brien TG, Rovera G: Tumor promoters: Effects on proliferation and differentiation of cells in culture. Life Sci 23:1979-1988, 1979

(10) Fiback E, Gambari R, Shaw PA, et al: Tumor promoter-mediated inhibition of cell differentiation: Suppression of the expression of erythroid functions in murine erythroleukemia cells. Proc Natl Acad Sci USA 76:1906-1910, 1979

(11) Shoyab J, DeLarco JE, Todaro GJ: Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. Nature 279:287-391, 1979

(12) Cole LJ, Nowell PC: Radiation carcinogenesis: The sequence of events. Science 150:1782-1789, 1965

(13) Farber E: Hyperplastic liver nodules. *In* Methods in Cancer Research, vol 7 (Busch H, ed). New York: Academic Press, 1973, pp 345-375

(14) _____: Carcinogenesis: Cellular evolution as a unifying thread: Presidential address. Cancer Res 33:2573-2550, 1973

(15) Teebor GW, Becker FF: Expression and persistence of hyperplastic nodules induced by *N*-2-fluorenylacetamide and their relationship to hepatocarcinogenesis. Cancer Res 31:1-3, 1971

(16) Becker FF: Alteration of hepatocytes by subcarcinogenic exposure to *N*-2-fluorenylacetamide. Cancer Res 35:1734-1736, 1975

(17) Peraino C, Fry RJ, Staffeldt E: Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res 31:1506-1512, 1971

(18) Ishidate M, Watanabe M, Odashima S: Effect of barbital on carcinogenic action and metabolism of 4-dimethylaminoazobenzene. Gan 58:267-281, 1976

(19) Kunz W, Schaude G, Thomas C: The effect of phenobarbital and halogenated hydrocarbons on nitrosamine carcinogenesis. Z Krebsforsch 72:291-304, 1969

(20) Schulte-Herman R: Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit Rev Toxicol 3:97-158, 1978

(21) Armuth V, Berenblum I: Systemic promoting action of phorbol in liver and lung carcinogenesis in AKR mice. Cancer Res 32:2259-2262, 1972

(22) Peraino C, Fry RJ, Staffeldt E: Enhancement of spontaneous hepatic tumorigenesis in C3H mice by dietary phenobarbital. J Natl Cancer Inst 51:1349-1350, 1973

(23) Thorpe E, Walker AT: The toxicology of dieldrin (HEOD). II. Comparative long-term oral toxicity studies in mice with dieldrin, DDT, phenobarbitone, B-BHC, and 2-BHC. Food Cosmet Toxicol 11:433-442, 1973

(24) Kitagawa T, Sugano M: Enhancing effect of phenobarbital on the development of enzyme-altered islands and hepatocellular carcinomas initiated by 3-methyl-4(dimethylamino) azobenzene or diethylnitrosamine. Gan 69:679-687, 1978

(25) Weisburger JH, Madison RM, Ward JM, et al: Modification of diethylnitrosamine liver carcinogenesis with phenobarbital but not with immunosuppression. J Natl Cancer Inst 54:1185-1188, 1975

(26) Pitot HC, Barsness L, Goldsworthy T, et al: Biochemical characterisation of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. Nature 271:456-458, 1978

(27) Peraino C, Fry RJ, Staffeldt E, et al: Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenyltrichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res 35:2884-2890, 1975

(28) Nishizumi M: Enhancement of diethylnitrosamine hepatocarcinogenesis in rats by exposure to polychlorinated biphenyls or phenobarbital. Cancer Lett 2:11-16, 1976

(29) Peraino C, Fry RJ, Staffeldt E, et al: Enhancing effects of phenobarbital and butylated hydroxytoluene on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Food Cosmet Toxicol 5:93-96, 1977

(30) Peraino C, Fry RJ, Grube DD: Drug-induced enhancement of hepatic tumorigenesis. *In* Carcinogenesis, Mechanisms of Tumor Promotion and Cocarcinogenesis, vol 2 (Slaga, TJ, Sivak A, Boutwell RK, eds). New York: Raven Press, 1978, pp 421-432

(31) Kitagawa T, Pitot MC, Miller EC, et al: Promotion by dietary phenobarbital of hepatocarcinogenesis by 2-methyl-*N,N*-dimethyl-4-aminoazobenzene in the rat. Cancer Res 39:112-115, 1979

(32) Pitot HC: Drugs as promoters of carcinogenesis. *In* The Induction of Drug Metabolism (Estabrook RW, Lindenlaub E, eds). New York: FK Schattauer-Verlag, 1979, pp 471-483

(33) Peraino C, Fry RJ, Staffeldt E, et al: Effects of varying the exposure to phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res 33:2701-2705, 1973

(34) Peraino C, Fry RJ, Staffeldt E: Effects of varying the onset and duration of exposure to phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis. Cancer Res 37:3623-3627, 1977

(35) Boutwell RK: Some biological aspects of skin carcinogenesis. Prog Exp Tumor Res 4:207-250, 1964

(36) Peraino C, Staffeldt E, Haugen DA, et al: Effects of varying the dietary concentration of phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis. Cancer Res 40:3268-3273, 1980

(37) Tannenbaum A, Silverstone M: The influence of the degree of caloric restriction the formation of skin tumors and hepatomas in mice. Cancer Res 9:724-727, 1949

(38) Tucker MJ: The effect of long-term food restriction on tumors in rodents. Int J Cancer 23:803-807, 1979

(39) Saffiotti U, Shubik P: The effects of low concentrations of carcinogen in epidermal carcinogenesis. A comparison with promoting agents. J Natl Cancer Inst 16:961-969, 1956

(40) Turosov V, Day N, Andrianov L, et al: Influence of dose on skin tumors induced in mice by single application of 7,12-dimethylbenz[*a*]anthracene. J Natl Cancer Inst 47:105-111, 1971

(41) Rossi L, Ravera M, Repetti G, et al: Long-term administration of DDT or phenobarbital-Na in Wistar rats. Int J Cancer 19:179-185, 1977

(*42*) Gold E, Gordis L, Tonascia J, et al: Increased risk of brain tumors in children exposed to barbiturates. J Natl Cancer Inst 61:1031–1034, 1978

(*43*) Clemmesen J, Hjalgrim-Jensen S: Is phenobarbital carcinogenic? A follow-up of 8,078 epileptics. Ecotoxicol Environ Safety 1:457–470, 1978

(*44*) Annegers JF, Kurland LT, Hauser WA: Brain tumors in children exposed to barbiturates. J. Natl Cancer Inst 63:3, 1979

(*45*) Gold EB, Gordis L, Tonascia JA, et al: Brain tumors in children exposed to barbiturates. J Natl Cancer Inst 63:3–4, 1979

Urinary Bladder Carcinogenesis with N-Substituted Aryl Compounds: Initiation and Promotion[1, 2]

Samuel M. Cohen[3, 4]

ABSTRACT—Aromatic amines have been implicated in the etiology of bladder cancer in humans since Rehn observed the disease in 3 workers in the German aniline dye industry in 1895. 2-Naphthylamine was identified 40 years later as one of the carcinogens in tests involving the feeding of the chemical to dogs. The discovery of *N*-2-fluorenylacetamide as a carcinogen in rodents inducing tumors of the bladder and other organs provided a more inexpensive and rapid model for the study of bladder carcinogenesis. The metabolic activation pathways of aromatic amine and amide compounds has been extensively examined. In the 1960's, organ-specific rodent models were discovered with the use of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, or *N*-methyl-*N*-nitrosourea. Recent experiments have demonstrated that bladder carcinogenesis can be divided into two stages similar to the initiation-promotion model in mouse skin. Possible promoters have included sodium saccharin, sodium cyclamate, and tryptophan. Certain metabolites of the latter compound are also N-substituted aryl compounds. Lastly, recent studies of the relationship of urine to the carcinogenic process in the bladder indicate that it can act as a promoting agent as well as a carrier of carcinogenic substances.—Natl Cancer Inst Monogr 58: 63–67, 1981.

In 1895, Rehn (*1*) reported the appearance of bladder cancer in workers in the dye industry and, in 1904, when reporting an additional 23 cases, he referred to a possible etiologic group of chemicals, ". . . aniline, its derivatives, and related compounds, such as naphthylamine, . . ." (*1*). These speculations were the initial indications that some aromatic amines might be carcinogenic and that human bladder cancer could be caused by chemicals. It was not until 1938 that the carcinogenicity of one of these chemicals was experimentally confirmed by Hueper in a non-human species, i.e. the induction of bladder cancer in dogs by 2-naphthylamine (*2*). Compared with the lack of success of previous investigators, the importance of the choice of species and the long time necessary for the induction of bladder cancer became evident. Although the dog has been useful for various studies, the long periods and enormous costs involved in such experiments have limited its usefulness (*1, 3, 4*). With the demonstration of bladder carcinogenicity by 2-FAA in the rat, a small animal experimental model became available, but its usefulness was limited by the number of other tissues in which neoplastic changes were found. Subsequently, 3 carcinogens with specificity toward the bladder in several species have been discovered: BBN administered in the drinking water (*5*); FANFT fed in the diet (*6*); and MNU instilled intravesically (*7*). The experimental models in dogs and rodents derived from these observations are widely used in studies of bladder carcinogenesis. The basis for model selection by an investigator depends on the experimental requirements and in some instances on adequacy of available facilities. Inasmuch as this Symposium concerns N-substituted aryl compounds, the remainder of this presentation will relate to various aspects of aromatic amine and nitro carcinogenesis. The epidemiology, metabolism, and tissue specificity will be described by others in this Symposium. I will limit my presentation to items specifically related to bladder carcinogenesis by these compounds.

CARCINOGENICITY OF N-SUBSTITUTED ARYL COMPOUNDS

Several aromatic amines are generally accepted as bladder carcinogens in humans including 2-naphthylamine, 4-aminobiphenyl, and benzidine (*1, 3, 4*). In addition, chlornaphazin (*1, 3, 4*), an alkylating agent used in cancer chemotherapy and several benzidine-derived azo dyes (*4*) also have been demonstrated to be human carcinogens, the parent aromatic amine of which is generated metabolically in vivo. 1-Naphthylamine does not appear to be a bladder carcinogen. The earlier reports concerning its carcinogenicity are most likely related to contamination by 2-naphthylamine. More recently, Johansson and colleagues (*8, 9*) observed that analgesic abuse induces papillary necrosis and interstitial nephritis in humans and, in addition, results in urothelial carcinomas not only in the renal pelvis but also in the ureters and bladder. Phenacetin, an aromatic acetamide, is present in these analgesic combinations and is probably the component most responsible for the carcinogenic effects based on analogy to known bladder carcinogens (*1, 3, 4*), metabolic data (*4*), and recent

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; BBN = *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; FANFT = N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; MNU = *N*-methyl-*N*-nitrosourea; AIC = 4-aminoimidazole-5-carboxamide.

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[2] Supported in part by Public Health Service grant CA15945 from the National Cancer Institute through the National Bladder Cancer Project.

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[4] I thank Drs. G. H. Friedell and R. E. Greenfield for their comments and advice.

evidence in rats that indicated it induced a low incidence of urothelial tumors (*10*). However, caffeine and either phenazone or aspirin are also in these analgesics and may enhance the carcinogenic action of phenacetin or they may be urothelial carcinogens, especially phenazone [(*10*); Johansson S: Personal communication].

As mentioned above, the aromatic amines identified as human carcinogens have also produced tumors in the bladders of experimental animals, particularly the dog (*1, 3, 4*). In rodents, these chemicals frequently induced urinary bladder tumors at lower rates than liver tumors and occasionally neoplasia in other tissues (*1, 3, 4*). Other aromatic amines which induce bladder tumors in one or more species include 2-methoxy-3-aminobenzofuran and some of the azo dyes such as 4-aminoazotoluene, 3,2′-dimethyl-4-aminobiphenyl, and 4-dimethylaminoazobenzene (*1, 3, 4,*). The nitro analogs of 2-naphthylamine and 2-fluorenamine are also bladder carcinogens, presumably by metabolic activation to the same intermediate, the *N*-hydroxyamine (*1, 3, 4*). Heterocyclic aromatic nitro compounds are also known bladder carcinogens in animals including FANFT, nitrofuran compounds closely related to FANFT (*11*), and a nitrothiazole compound, niridazole, which is used for the treatment of schistosomiasis in humans (*12*). However, incidence did not increase when niridazole was administered to mice or hamsters infected by intravesical installation of *Schistosoma mansoni* (*13*).

METABOLIC ACTIVATION

Activation of aromatic amine (and amide) and aromatic nitro carcinogens is thought by many to involve the N-hydroxy intermediate (*4*). Apparently for liver carcinogenesis, although esterification with sulfate is a necessary additional step (*14*), sulfation is not related to bladder carcinogenesis. Whereas the ultimate form of these carcinogens for the bladder is unknown, various possibilities have been suggested. The first problem encountered with any of these chemicals is for one to determine in which form the carcinogen is delivered by the urine to the bladder; the compound must be stable enough not to be degraded in the process. The second problem is to detect the mechanism by which it is converted to a reactive ultimate form. N-hydroxylation of the aromatic amine carcinogens occurs in the liver and is transported to the urine as the glucuronide. Recently, Kadlubar et al. (*15*) demonstrated the formation of the *N*-glucuronides of aromatic *N*-hydroxylamine carcinogens which are stable above pH 7, rather than the *O*-glucuronides. The *N*-glucuronides can be transported through the blood but are unstable in urine below pH 5; cleavage of the glucuronides yields the aromatic *N*-hydroxylamines (*15*). Conversion of the latter to an arylnitrenium ion either directly or after additional esterification has been suggested by the finding of covalent binding to tissue macromolecules (*15, 16*).

An alternate mechanism for delivery of an aromatic hydroxylamine to the urine is the generation of the metabolite immediately before urine is formed and delivered into the renal calices. This would require the metabolic step to take place in the collecting ducts of the renal medulla. Zenser and co-workers (*17*) demonstrated that cyclooxygenase, which is involved in the synthesis of prostaglandins and requires oxygen and arachidonic acid, is present at high levels in the collecting ducts and is capable of oxidizing benzidine.

Yet another possibility, though seemingly more remote and limited to specific situations, involves the generation of a diazo ion from the aromatic amine plus nitrite in an acid medium similar to nitrosation of secondary amines that yield *N*-nitrosamines. The urine frequently has an acid pH, with nitrite also present, particularly in bacterial cystitis. Although diazotization of known carcinogenic amines has not been demonstrated in urine, the diazo ion of AIC, a normal constituent of urine derived from the de novo purine biosynthetic pathway, appeared in rat urine after the oral administration of AIC and sodium nitrite (*18*). Whereas a few tumors occurred in female rats after oral or ip administration of the diazo form of AIC, including 1 bladder tumor (*19*), its carcinogenicity and the role of diazo ions in aromatic amine bladder carcinogenesis remain uncertain.

Apparently, the carcinogenicity of aromatic amines for the bladder is species related and involves N-hydroxylation (*4*), acetylation, and deacetylation (*20, 21*). Failure to N-hydroxylate these compounds explains the lack of carcinogenic activity of these compounds in the guinea pig bladder (*4*). Aromatic amines, when compared with acetamides, are likely to have greater specificity for the bladder than the liver in species which are incapable of acetylating the amine or deacetylating the acetamide. Thus aromatic amines are not acetylated in the dog, whereas they are in rodents (*20*). The acetamide of 4-aminobiphenyl is more carcinogenic toward the bladder in dogs than is the acetamide of 2-fluorenamine, but 2-acetylaminonaphthalene does not induce tumors in this animal (*4, 21*). The rates of deacetylation by dog liver is high for 4-acetylaminobiphenyl and low for 2-acetylaminonaphthalene (*21*). In humans, acetylation is genetically controlled with a bimodal distribution separating people into 2 groups as fast and slow acetylators. The relationship of this genetic trait to the susceptibility to aromatic amine carcinogenesis is under investigation by Lower and Bryan and their colleagues (*22*).

Aromatic nitro compounds can also be converted to *N*-hydroxylamines by nitroreduction in the liver, but this can also occur in most other tissues (*23*). In addition, numerous enzyme systems can perform the reduction including mixed function oxidase, xanthine oxidase (*24*), and the cyclooxygenase system (*25*) mentioned above for benzidine. The former two enzyme systems require anaerobic conditions, the latter requires oxygen; which of them is important in carcinogenesis has not been ascertained. However, the urothelium is clearly capable of nitroreduction (*23, 24*).

FANFT-INDUCED CARCINOGENESIS

Several nitrofurans have been demonstrated to be carcinogenic in various species (*26*), but only FANFT (*27*) has been potent in the urinary bladder in most species studied, including the rat (*6*), mouse (*7*), hamster (*11*), and dog (*28*). Other nitrofurans also induced bladder cancer in

hamsters (*11*), but in guinea pigs, none of the nitrofurans tested demonstrated carcinogenic activity toward the bladder or any other tissues (*29*). Although the original description of FANFT carcinogenicity was in Sprague-Dawley rats, we used F344 rats in our experiments (*30*) and found no differences between males and females (*31*); however, a dose response was observed (*32*).

When FANFT is fed as 0.2% of the diet, it follows a sequence (*30, 31*) similar to that found with other urinary bladder carcinogens beginning with a simple hyperplasia after 2–4 weeks that increases in severity through 8–10 weeks, when nodular and papillary hyperplasia are evident. Papillomas appear after 14–20 weeks and noninvasive carcinomas are present by 25 weeks. Microinvasion through the basement membrane then becomes evident with deep invasion first seen after 40 weeks. All rats fed 0.2% FANFT for 26 or more weeks die of bladder cancer by the end of 80 weeks, occasionally with metastases and/or hydronephrosis. Those given this same amount for 6 weeks or less had lesions which disappeared within 4 weeks after FANFT was discontinued, and the bladders remained normal through 52 (*33*) and 84 (*34*) weeks, but a few carcinomas were noted in such rats when they were studied for 2 years (*35*). When 0.2% FANFT is administered for 8 or 10 weeks followed by a control diet, rats show partial regression of the bladder lesions but eventually most develop urinary bladder cancer. Fed at this same dosage for 12 or more weeks, FANFT induces progressive lesions which result in bladder cancer in all of the rats.

INITIATION-PROMOTION

The above studies involved the administration of a single carcinogen which induced bladder cancer. Ito and his colleagues (*36*) demonstrated that the simultaneous administration of low doses of more than 1 bladder carcinogen also results in the induction of bladder cancer, even if the dose of each carcinogen was insufficient to induce cancer; they used BBN, FANFT, 2-FAA, and 3,3′-dichlorobenzidine. They also showed that these same carcinogens administered sequentially rather than simultaneously had a similar synergistic effect (*37*). However, these studies involved complete bladder carcinogens.

During the past 10 years, the carcinogenesis model of initiation and promotion, first described in mouse skin (*38*), occurred in other tissues and species including the rat bladder. Hicks (*39*) discovered that a subcarcinogenic dose of MNU can act as an initiator in rats with subsequent administration of either sodium saccharin or sodium cyclamate as promoters. Fed as 0.2% of the diet for 6 weeks to male Fischer rats, FANFT can act as an initiator with sodium saccharin or DL-tryptophan as promoters (*35*). Promoting activity was evident whether the agent was administered immediately after initiation or after a 6-week delay when the rats received no treatment. In experiments with rats not yet completed, FANFT administered for 4 weeks seems to act as an initiator, and the low incidence of carcinomas that occurs when FANFT was used for 6 weeks is eliminated; L-tryptophan acts as a promoter (unpublished observations). L-Tryptophan has also been shown to have promoting activity in mice after FANFT initiation (*40*), whereas DL-tryptophan has shown such activity in dogs after 4-aminobiphenyl or 2-naphthylamine initiation (*41*). Also, sodium saccharin given after BBN recently resulted in high incidences of bladder cancer (Ito N: Personal communication).

Although sodium saccharin induces a low incidence of bladder cancer in rats when administered without other chemicals, particularly if given through two generations (*42*), it has not generally induced bladder hyperplasia as frequently as one would expect for promoting agents. However, using the techniques of autoradiography and scanning electron microscopy, we recently detected that mild, multifocal hyperplasia could be observed in all rats fed sodium saccharin (unpublished observations).

Tryptophan has had a long history of association with bladder cancer in experimental animals and in humans beginning with the report of Dunning et al. (*43*) who found that tryptophan increased the bladder carcinogenicity of 2-FAA in rats. In varying percentages of patients with bladder cancer, urinary tryptophan metabolites are elevated (*1, 44*), and this abnormality apparently can be corrected by the administration of vitamin B_6. Some of these metabolites are aromatic amines: anthranilic acid, kynurenine, and their ortho-hydroxylated analogs. The mechanisms by which tryptophan and/or its metabolites act as promoters remain speculative, but in keeping with the properties expected of promoters, these compounds appear to be nonmutagenic (*45*).

Lastly, in dealing with bladder carcinogenesis, one must explain the function of urine. Inasmuch as bladder carcinogens reach the bladder through the urine rather than the blood (*46*), might urine not be more directly involved? The results of recent provocative experiments indicated that normal urine and/or its components act as promoters for bladder carcinogenesis. Hashimoto (*47*) has induced transformation of rat bladder epithelial cells in tissue culture by using BBN or its analogs, but this action happened only if urea was also present. Rats fed FANFT for 14 weeks and then given control diets developed bladder carcinomas (*48*). However, if the urinary flow is diverted away from the bladder by a bilateral ureterosigmoidostomy, carcinoma does not develop!

REFERENCES

(*1*) PRICE JM: Etiology of bladder cancer. *In* Benign and Malignant Tumors of the Urinary Bladder (Maltry E, ed). Flushing, N.Y.: Medical Examination, 1971, pp 185–251

(*2*) HUEPER WC, WILEY FH, WOLFE HD: Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. J Ind Hyg Toxicol 20:46–91, 1938

(*3*) CLAYSON DB, COOPER EH: Cancer of the urinary tract. Adv Cancer Res 13:271–381, 1970

(*4*) RADOMSKI JL: The primary aromatic amines: Their biological properties and structure-activity relationships. Annu Rev Pharmacol Toxicol 19:129–157, 1979

(*5*) DRUCKREY H, PREUSSMANN R, IVANKOVIC S, et al: Selektive enzengung von Blasenkrebs an Ratten durch Dibutyl-und N-butanol (4) nitrosamin. Z Krebsforsch 66: 280–290, 1964

(*6*) ERTURK E, PRICE JM, MORRIS JE, et al: The production of

carcinoma of the urinary bladder in rats by feeding N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. Cancer Res 27:1998—2002, 1967

(7) Hicks RM, Wakefield JStJ: Rapid induction of bladder cancer in rats with *N*-methyl-*N*-nitrosourea. I. Histology. Chem Biol Interact 5:139–152, 1972

(8) Johansson S, Angervall L. Bengtsson U, et al: Uroepithelial tumors of the renal pelvis associated with abuse of phenacetin-containing analgesics. Cancer 33: 743–753, 1974

(9) Johansson S, Wahlqvist L: Tumors of urinary bladder and ureter associated with abuse of phenacetin-containing analgesics. Acta Pathol Microbiol Scand [A] 85: 768–774, 1977

(10) Johansson S, Angervall L: Carcinogenicity of phenacetin. Science 204:130, 1979

(11) Croft WA, Bryan GT: Production of urinary bladder carcinomas in male hamsters by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, N-[4-(5-nitro-2-furyl)-2-thiazolyl] acetamide, or formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide. J Natl Cancer Inst 51:941–949, 1973

(12) Bulay O, Clayson DB, Shubik P: Carcinogenic effects of niridazole in rats. Cancer Lett 4:305–310, 1978

(13) Bulay O, Urman H, Clayson DB, et al: Carcinogenic effects of niridazole on rodents infected with *Schistosoma mansoni*. J Natl Cancer Inst 59:1625–1630, 1977

(14) DeBaun JR, Miller EC, Miller JA: *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methion-*S*-yl) binding in rat liver. Cancer Res 30:577–595, 1970

(15) Kadlubar FF, Miller JA, Miller EC: Hepatic microsomal N-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res 37:805–814, 1977

(16) Kadlubar FF, Miller JA, Miller EC: Guanyl O^6-arylamination and O^6-arylation of DNA by the carcinogen *N*-hydroxy-1-naphthylamine. Cancer Res 38:3628–3638, 1978

(17) Zenser TV, Mattammal MB, Davis BB: Cooxidation of benzidine by renal medullary prostaglandin cyclooxygenase. J Pharmacol Exp Ther 211:460–464, 1979

(18) Lower GM Jr, Lanphear SP, Johnson BM, et al: Aryl and heterocyclic diazo compounds as potential environmental electrophiles. J Toxicol Environ Health 2:1095–1107, 1977

(19) Beal DD, Skibba JL, Croft WA, et al: Carcinogenicity of the antineoplastic agent, 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide, and its metabolites in rats. J Natl Cancer Inst 54:951–957, 1975

(20) Lower GM, Jr, Bryan GT: Enzymatic N-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. Biochem Pharmacol 22:1581–1588, 1973

(21) _____: Enzymic deacetylation of carcinogenic arylacetamides by tissue microsomes of the dog and other species. J Toxicol Environ Health 1:421–432, 1976

(22) Lower GM Jr, Nilsson T, Nelson CE, et al: *N*-Acetyltransferase phenotype and risk in urinary bladder cancer: Approaches in molecular epidemiology. Environ Health Perspect 29:71–79, 1979

(23) Symms KG, Juchau MR: Mechanisms of aromatic nitrogroup reduction in the soluble fraction of human placenta. Biochem Pharmacol 21:2519–2527, 1972

(24) Swaminathan S, Lower GM Jr: Biotransformations and excretion of nitrofurans. *In* Carcinogenesis—A Comprehensive Survey: Nitrofurans, vol 4 (Bryan GT, ed). New York: Raven Press, 1978, pp 59–98

(25) Zenser TV, Mattammal MB, Davis BB: Metabolism of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide by prostaglandin endoperoxide synthetase. Cancer Res 40:114–118, 1980

(26) Cohen SM: Toxicity and carcinogenicity of nitrofurans. *In* Carcinogenesis—A Comprehensive Survey: Nitrofurans, vol 4 (Bryan GT, ed). New York: Raven Press, 1978, pp 171–231

(27) Erturk E, Cohen SM, Bryan GT: Urinary bladder carcinogenicity of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide in female Swiss mice. Cancer Res 30:1309–1311, 1970

(28) Erturk E, Atassi SA, Yoshida O, et al: Comparative urinary and gallbladder carcinogenicity of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide and N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide in the dog. J Natl Cancer Inst 45:535–542, 1970

(29) Croft WA, Skibba JL, Lower GM Jr, et al: Refractoriness of guinea pigs to carcinogenesis by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT), N-[4-nitro-2-furyl)-2-thiazolyl]acetamide (NFTA), formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide (FNT) and 4 (5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide (DTIC, NSC-45388). Proc Am Assoc Cancer Res 17: 122, 1976

(30) Tiltman AJ, Friedell GH: The histogenesis of experimental bladder cancer. Invest Urol 9:218–226, 1971

(31) Cohen SM, Friedell GH: Carcinoma of the urinary bladder induced in Fischer rats by N-[4-(5-nitro-2-furyl)-2-thiazolyl[formamide. Am J Pathol 95:849–852, 1979

(32) Arai M, Cohen SM, Jacobs JB, et al: Effect of dose on urinary bladder carcinogenesis induced in F344 rats by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. J Natl Cancer Inst 62:1013–1016, 1979

(33) Cohen SM, Jacobs JB, Arai M, et al: Early lesions in experimental bladder cancer: Experimental design and light microscopic findings. Cancer Res 36:2508–2511, 1976

(34) Jacobs JB, Arai M, Cohen SM, et al: A long-term study of reversible and progressive urinary bladder cancer lesions in rats fed N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. Cancer Res 37:2817–2821, 1977

(35) Cohen SM, Arai M, Jacobs JB, et al: Promoting effect of saccharin and DL-tryptophan in urinary bladder carcinogenesis. Cancer Res 39:1207–1217, 1979

(36) Tsuda H, Miyata Y, Murasaki G, et al: Synergistic effect of urinary bladder carcinogenesis in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, *N*-2-fluorenylacetamide, and 3,3′dichlorobenzidine. Gan 68:183–192, 1977

(37) Tatematsu M, Miyata Y, Mizutani M, et al: Summation effect of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, *N*-2-fluorenylacetamide, and 3,3′-dichlorobenzidine on urinary bladder carcinogenesis in rats. Gan 68:193–202, 1977

(38) Scribner JD, Suss R: Tumor initiation and promotion. Int Rev Exp Pathol 18:138–198, 1978

(39) Hicks RM, Chowaniec J: The importance of synergy between weak carcinogens in the induction of bladder cancer in experimental animals and humans. Cancer Res 37:2943–2949, 1977

(40) Matsushima M: The role of the promoter L-tryptophan on tumorigenesis in the urinary bladder. 2. Urinary bladder carcinogenicity of FANFT (initiating factor) and L-tryptophan (promoting factor) in mice. Jpn J Urol 68:731–736, 1977

(41) Radomski JL, Radomski T, MacDonald WE: Cocarci-

nogenic interaction between D,L-tryptophan and 4-aminobiphenyl or 2-naphthylamine in dogs. J Natl Cancer Inst 58:1831–1834, 1977

(*42*) Office of Technology Assessment: Cancer Testing Technology and Saccharin. Washington D.C.: U.S. Govt Print Off, 1977

(*43*) Dunning WF, Curtis MR, Maun ME: The effect of added dietary tryptophan on the occurrence of 2-acetylaminofluorene-induced liver and bladder cancer in rats. Cancer Res 10:454–459, 1950

(*44*) Bryan GT: The role of urinary tryptophan metabolites in the etiology of bladder cancer. Am J Clin Nutr 24: 841–847, 1971

(*45*) Bowden JP, Chung KT, Andrews AW: Mutagenic activity of tryptophan metabolites produced by rat intestinal microflora. J Natl Cancer Inst 57:921–924, 1976

(*46*) Scott WW, Boyd HL: Study of the carcinogenic effect of beta-naphthylamine on the normal and substituted isolated sigmoid loop bladder of dogs. J Urol 70:914–925, 1953

(*47*) Hashimoto Y, Kitagawa HS: In vitro neoplastic transformation of epithelial cells of rat urinary bladder by nitrosamine. Nature 252:497–499, 1974

(*48*) Oyasu R, Rowland RG, Hirao Y, et al: Growth-stimulating effect of normal urine on the rat bladder epithelium. Proc Am Assoc Cancer Res 20:100, 1979

Activation of Certain *N*-Arylacetamides and *N*-Arylacetohydroxamic Acids in Relation to Mammary Gland Tumorigenesis in the Rat [1, 2]

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ABSTRACT—This report describes activation of certain *N*-arylacetamides and *N*-arylacetohydroxamic acids and its relationship to mammary gland tumorigenesis. Evidence is presented that metabolic activation of *N*-2-fluorenylacetamide (2-FAA) by mixed function oxidase of liver microsomes is the primary requirement for tumor induction in the mammary gland by this compound in young adult female rats. Mammary gland microsomes of those rats appear incapable of N-hydroxylating 2-FAA. Mammary gland microsomes of lactating rats, however, are capable of converting small amounts of 2-FAA to *N*-hydroxy-2-FAA, which suggests that the ability to perform certain metabolic activation reactions may depend on the stage of development of the mammary gland which is hormonally regulated. According to a current theory of chemical carcinogenesis, *N*-arylacetohydroxamic acids would have to be activated to electrophilic reactants to become ultimate carcinogens. Three mechanisms by which such reactants could be generated from *N*-arylacetohydroxamic acids in the mammary gland are reviewed: 1) nonenzymatic acetylation; 2) enzymatic N-O-acetyl transfer to form *N*-acetoxyarylamines; 3) one-electron oxidation to nitroxyl free radicals. In addition, the potential role of the metabolically formed glucuronide of *N*-hydroxy-2-FAA in mammary gland tumorigenesis is discussed.—Natl Cancer Inst Monogr 58: 69–77, 1981.

Tumor induction in the mammary gland of female rats is the most prevalent result of exposure to a variety of chemical compounds. The high incidence of breast cancer in women may be the result of a similar high susceptibility of the human mammary gland to the action of endogenous and exogenous carcinogens. In rats, mammary gland tumors can be induced with polycyclic aromatic hydrocarbons (*1, 2*), aromatic amines and amides (*3–5*), and alkylating agents (*6*). Numerous chemical compounds (procarcinogens) undergo metabolic activation to become proximate and ultimate carcinogens (*7*). Liver, by virtue of its capacity for metabolism of foreign compounds, is capable of activation of procarcinogens to carcinogens. However, the carcinogenic metabolites generated therein do not necessarily initiate a carcinogenic process at this site. Whether "extrahepatic targets," such as the mammary gland, are capable of metabolizing carcinogens in situ is a problem that has been investigated only in recent years. My efforts and those of other investigators directed at elucidating mammary tumorigenesis by certain *N*-arylacetamides and related compounds which require metabolic activation to exert their carcinogenic properties are reviewed.

ACTIVATION OF *N*-ARYLACETAMIDES THROUGH N-HYDROXYLATION

The metabolic conversion of 2-FAA to *N*-OH-2-FAA, a reaction discovered in the Millers' laboratory nearly 20 years ago, has since been proposed as the primary activation reaction in carcinogenesis by 2-FAA (*8, 9*). The same type of metabolic activation was reported for 4-acetylaminobiphenyl (*10*). This led Gutmann and associates (*11, 12*) to investigate whether N-hydroxylation is a general requirement for arylamide carcinogenesis. In his laboratory, several arylamides and the corresponding N-hydroxy compounds in the fluorenyl, biphenylyl, and phenyl series were synthesized and tested for carcinogenicity in the rat by the ip route (*12*). The arylamides were only weakly carcinogenic, whereas their respective N-hydroxy derivatives in the fluorenyl and biphenylyl series were potent mammary gland carcinogens with a standard compound, *N*-OH-2-FAA, which exhibited the highest ratio of malignant to benign neoplasms. Recently, we induced mammary gland tumors with a single ip injection of *N*-OH-2-FAA in 51-day-old Sprague-Dawley rats (table 1). Rats at about this age are considered the most susceptible to mammary tumor induction (*2, 13*). The single dose of *N*-OH-2-FAA (0.16 mmol/rat) equaled the total dose given in 12 ip injections during a 4-week period and produced an equally high incidence of mammary gland tumors. However, the

Abbreviations: 2(or 3)-FAA = *N*-2(or 3)-fluorenylacetamide; *N*-OH-2(or 3)-FAA = *N*-hydroxy-2(or 3)-fluorenylacetamide; 3-MCA = 3-methylcholanthrene; *N*-AcO-2(or 3)-FAA = *N*-acetoxy-2(or 3)-fluorenylacetamide; *N*-OH-2-FA = *N*-2-fluorenylhydroxylamine; *N*-OGl-2-FAA = glucuronide of *N*-OH-2-FAA.

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TABLE 1.—*Comparison of carcinogenicity of* N-*OH-2-FAA after single or multiple ip injections to female Sprague-Dawley rats*

Compound administered [a]	Average total dose/rat, mmol	No. of injections	Age of rats at the onset of treatment, days	No. of rats with tumors/ No. of rats used	Mammary tumors No.	Type	No. of rats with this tumor	Mean latent period, mo
Vehicle	—	1	51	0/12		—		—
N-OH-2-FAA	0.16	1	51	13/15[b]	13	Adenocarcinomas	8	3.7
					9	Fibroadenomas	6	8.8
					2	Adenomas	2	4.0
Vehicle	—	12	39	2/12	1	Fibroadenoma		8.0
					1	Adenoma		11
N-OH-2-FAA	0.16	12	39	15/15	27	Adenocarcinomas	13	2.8
					5	Fibroadenomas	2	3.8
					8	Adenomas	4	3.1
N-OH-2-FAA	0.17	12	40	11/12	19	Adenocarcinomas	8	2.4
					5	Fibroadenomas	5	2.6
					3	Adenomas	2	2.8
					(1 ear duct tumor)			8.0

[a] The compound (21.8 or 10.9 mg) was suspended in a vehicle consisting of 7% gum arabic in 0.9% NaCl and given once or 12 times at a dose level of 1.0 or 0.1 mmol/kg body wt, respectively.

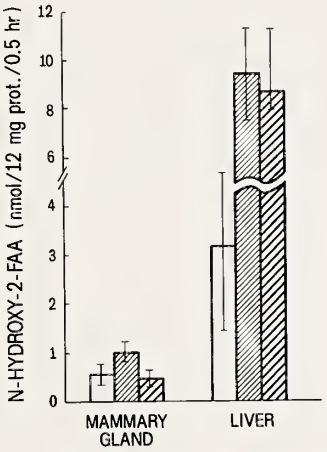
[b] After a single intragastric dose of 0.42 mmol *N*-OH-2-FAA, 4 of 15 rats developed mammary adenocarcinomas (*4*).

mean latent period of malignant tumors after the single dose of carcinogen was delayed by about a month, and the multiplicity of these tumors was lower than after multiple ip injections (1.6/rat vs. 2.3/rat, respectively). The results of these tests reemphasized the potency of *N*-OH-2-FAA as a mammary gland carcinogen.

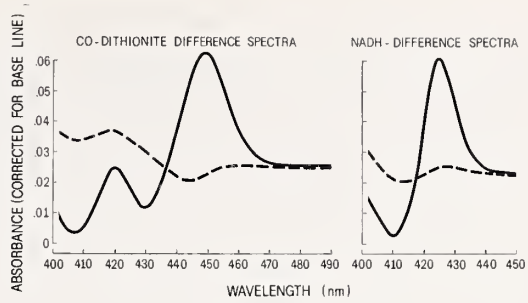
Because systemic administration of the compound gives no indication as to the site of its metabolic activation and the structure of the reactive metabolite, we applied the *N*-fluorenylacetamides and the corresponding N-hydroxy compounds topically to investigate whether N-hydroxylation of the arylamide is required for mammary tumorigenesis. In these tests, we applied a single dose (0.02 mmol) of each compound as a solid to the left thoracic mammary glands (*14, 15*). Whereas 2-FAA and 3-FAA were only weakly carcinogenic by this route, the corresponding hydroxamic acids, *N*-OH-2-FAA and *N*-OH-3-FAA, induced a high incidence of tumors at the site of application. The differences in carcinogenicities between these compounds were also apparent from the mean latent periods of malignant tumors which for *N*-fluorenylacetamides were twice as long as for *N*-fluorenylacetohydroxamic acids. The results of these tests indicated that N-hydroxylation of the *N*-arylacetamides is essential for their carcinogenicity in the mammary gland. In addition, they suggested that the mammary gland does not convert much of the *N*-arylacetamides to the N-hydroxy compounds. Inasmuch as N-hydroxylation of 2-FAA in rat liver is catalyzed by microsomal mixed function oxidase (*16–18*), we examined the capacity of mammary gland microsomes for N-hydroxylation of *N*-fluorenylacetamides and compared it with that of the liver (*14, 15*). In contrast to liver microsomes, mammary gland microsomes of the young adult female rats appeared devoid of 2-FAA N-hydroxylating activity. Pretreatment of these rats with 3-MCA, an inducer of the hepatic catalyst (cytochrome $P_{1\text{-}450}$) of N-hydroxylation of 2-FAA, failed to induce either the cytochrome or 2-FAA N-hydroxylating activity in the mammary gland microsomes (*15*). Mammary gland microsomes of young adult female rats also appeared to lack 3-FAA N-hydroxylating activity (*14*). Liver microsomes of rats either untreated or treated with 3-MCA yielded only trace amounts of *N*-OH-3-FAA from 3-FAA (*17*). These findings suggested that only *N*-arylacetamides that are N-hydroxylated to appreciable levels in the liver would be carcinogenic for the mammary gland after systemic administration.

Recently, we demonstrated a novel type of induction of N-hydroxylation of 2-FAA in rat liver microsomes after acute or chronic treatment of rats with this compound (*18*). This induction was different from the induction by 3-MCA in that it did not involve cytochrome $P_{1\text{-}450}$. Therefore, we investigated whether chronic treatment of rats with 2-FAA would also induce its N-hydroxylation in the mammary gland. In these experiments, we used lactating rats which were treated throughout a 3-week lactation period with 2-FAA (text-fig. 1). At the end of lactation, the minor amounts of *N*-OH-2-FAA produced by the mammary gland microsomes of untreated rats were not significantly increased in the treated rats. By contrast, in the liver microsomes of these animals, N-hydroxylation of 2-FAA was significantly increased. Spectral determination of the dithionite-reduced carbon monoxide adduct of cytochrome P_{450} showed that, in contrast to liver microsomes, mammary gland microsomes of lactating rats and those of young adults studied earlier (*15*) lacked cytochrome P_{450} (text-fig. 2). Instead, these microsomes invariably exhibited a peak, attributed to cytochrome P_{420} and contained small amounts of cytochrome b_5. These results are in agreement with those of Bruder et al. (*19*) who, with the use of spectrophotometric and immunologic methods, detected the presence of a b-type cytochrome in the rat mammary gland that consisted of cytochrome b_5, apparently identical with that of liver microsomes, and possibly 2 forms of cytochrome P_{420} that differed in carbon monoxide binding capacity. These investigators also did not detect cytochrome P_{450} in mammary gland

microsomes of lactating rats. However, they reported a low NADPH-dependent cytochrome c (P_{450}) reductase activity, which we failed to detect in this tissue (table 2). The data suggested that the enzyme system chiefly responsible for N-hydroxylation of 2-FAA in the liver may be different from that present in the mammary gland.



TEXT-FIGURE 1.—Effect of chronic treatment of 11 lactating rats with 2-FAA on N-hydroxylation of 2-[1′-^{14}C]FAA in the mammary gland and liver microsomes. 2-FAA was suspended in 7% gum arabic in 0.9% NaCl at a concentration of 20 mg/ml and injected ip 10 times during lactation at a dose level of 0.2 or 0.3 mmol/kg body wt; the average cumulative dose of 2-FAA was 0.55 (*narrow-hatched bars*) or 0.89 mmol/rat (*wide-hatched bars*), respectively. Composition of the incubation system and assay of N-hydroxylation of 2-[1′-^{14}C]FAA were described in (*18*). The differences in N-hydroxylation of 2-FAA: 1) by mammary gland microsomes of untreated and 2-FAA-treated rats were not significant; 2) by liver microsomes of untreated and 2-FAA-treated rats ($P<0.005$) were significant; and 3) by mammary gland and liver microsomes of untreated rats ($P<0.05$) and of 2-FAA-treated rats ($P<0.001$) were significant. *Open bars* = untreated rats.



TEXT-FIGURE 2.—Difference spectra of liver (——) and mammary gland microsomes (----) from a lactating rat.

In conclusion, our data support the requirement for N-hydroxylation of the *N*-arylacetamide in mammary gland tumorigenesis. However, in young adult female rats, which are normally used for carcinogenicity tests because of their susceptibility to tumor induction, the activation of the carcinogen appears to occur predominantly in the liver. Further support for this view comes from our recent studies with disulfiram, a known modifier of chemical carcinogenesis (*22*). Disulfiram, an antioxidant containing the thiono-sulfur group, has suppressed tumor formation induced by certain carcinogens at various sites. This compound is a nonspecific inhibitor of liver microsomal enzymes and may, therefore, affect metabolic activation of carcinogens. Accordingly, we examined the effect of dietary intake of disulfiram on mammary gland tumorigenesis by 2-FAA and *N*-OH-2-FAA (*23*). Disulfiram given in the diet at a concentration of 0.9% for 1 week before and throughout the carcinogen treatment (0.1 mmol/kg body wt) three times a week for 4 weeks reduced the incidence of mammary tumors induced with 2-FAA by 50% and extended the mean latent period of malignant tumors from 5 to 10 months. However, this antioxidant had no effect on mammary tumorigenesis by *N*-OH-2-FAA. These results suggested that disulfiram interferes

TABLE 2.—*Effect of chronic treatment of lactating rats with 2-FAA on microsomal enzymes of the liver and mammary gland* [a]

Microsomal enzyme[b]	Liver			Mammary gland		
	Untreated	2-FAA-treated, mmol/rat [c]		Untreated	2-FAA-treated, mmol/rat [c]	
		0.55	0.89		0.55	0.89
Cytochrome P_{450}[d]	0.31 ± 0.11	0.47 ± 0.13	0.29 ± 0.07	ND[e]	ND	ND
NADPH-cytochrome c (P_{450}) reductase[f]	28.4 ± 4.1	47.1 ± 16.4	35.4 ± 7.6	ND[g]	ND	ND
Cytochrome b_5[d]	0.22 ± 0.04	0.30 ± 0.06	0.25 ± 0.04	0.032 ± 0.014	0.025 ± 0.005	0.047 ± 0.025
Total heme[h]	0.92 ± 0.25	1.13 ± 0.08	0.99 ± 0.18	0.28 ± 0.13	0.21 ± 0.08	0.18 ± 0.07

[a] The values are the means ± SD. The differences between values of untreated and 2-FAA-treated rats were not significant (*t* test). ND = not detectable.

[b] Unit of measurement is nanomole(s)/milligram protein except NADPH-cytochrome c (P_{450}) reductase, which is nanomoles cytochrome c reduced/milligram protein/minute.

[c] Treatment regimen is described in the legend of text-figure 1.

[d] Method of Omura and Sato (*20*) was used in the determinations.

[e] Limit of detection was 0.02 nmol/mg protein.

[f] Values were determined as described in (*18*).

[g] Limit of detection was 5.4 nmol/mg protein/min.

[h] Values were determined by Falk's method (*27*).

with the activation of 2-FAA to the N-hydroxy compound, possibly through an effect on cytochrome P_{450} of hepatic mixed function oxidase (*24*). Subsequently, we found that N-hydroxylation of 2-FAA was significantly inhibited in hepatic microsomes by disulfiram given orally to untreated rats and those given 2-FAA (*23*). A similar inhibition was shown in vitro after preincubation of hepatic microsomes with disulfiram. Measurements of cytochrome P_{450} after pretreatment of rats or microsomes with the inhibitor showed no appreciable changes in this hemoprotein content. Therefore, disulfiram apparently inhibits N-hydroxylation of 2-FAA in rat liver by a mechanism(s) other than depression of the cytochrome P_{450} level. Because both the substrate and the inhibitor bind to cytochrome P_{450} and produce a type I spectrum, we suggested that disulfiram may interfere with the binding of 2-FAA and thus alter its metabolism. The lack of an inhibitory effect of disulfiram on mammary tumor induction by *N*-OH-2-FAA suggests that it does not interfere with the metabolism of *N*-OH-2-FAA to an ultimate carcinogen.

ACTIVATION OF *N*-ARYLACETOHYDROXAMIC ACIDS TO ELECTROPHILIC REACTANTS

According to the current theory of chemical carcinogenesis proposed and advanced by the Millers, ultimate agents that initiate neoplasia are electrophilic reactants capable of interacting with nucleophilic sites in critical cellular macromolecules (*7*). Because *N*-arylacetohydroxamic acids, such as *N*-OH-2-FAA, are not electrophilic per se and do not react in nonenzymatic systems with proteins or nucleic acids (*25, 26*), their further biotransformation to potentially electrophilic species has been postulated (*27*). With the topically active mammary gland carcinogens, *N*-OH-2-FAA and *N*-OH-3-FAA, one can reasonably assume that their activation takes place in the target tissue. In the rat mammary gland, three types of activation of *N*-arylacetohydroxamic acids to potentially electrophilic reactants have been investigated in recent years: 1) esterification to esters capable of spontaneous decomposition to the electrophilic arylamidonium ion; 2) N–O-acetyl transfer to form *N*-acetoxyarylamines that yield the electrophilic arylnitrenium ion, and 3) one-electron oxidation to nitroxyl radicals that are weakly electrophilic per se and are capable of dismutating to potentially electrophilic reactants. Except for acetate esters of *N*-arylacetohydroxamic acids, the other potentially electrophilic compounds are too reactive to be isolated from the incubation systems and, therefore, their transient existence was detected by their entrapment with various nucleophiles, such as *N*-acetylmethionine, guanosine, or tRNA. Their transient existence in vivo was deduced from the interaction products with nucleophilic sites of cellular macromolecules, such as methionyl or guanosyl residues in proteins or nucleic acids. The nature of the reactive species was inferred from the type of products formed. Thus the products arising from the interaction of the arylamidonium ion (or its resonance forms) with nucleophiles retain the N-acetyl group of the parent *N*-arylacetohydroxamic acids, whereas the products arising from the interaction of the arylnitrenium ion (or its resonance forms) with nucleophiles are devoid of the N-acetyl group.

Sulfate and acetate esters of *N*-OH-2-FAA were first considered as possible sources of the arylamidonium ion in the mammary gland. However, this tissue lacks *N*-OH-2-FAA sulfotransferase activity (*28*), which precludes the formation of *N*-sulfonoxy-2-FAA and its macromolecular adducts after topical administration of the carcinogen (*14*). The small amounts of *N*-sulfonoxy-2-FAA formed in the livers of female Sprague-Dawley rats after systemic administration of *N*-OH-2-FAA (*29, 30*) and the high reactivity of this ester argue against its transport to the mammary gland. Thus the mechanism by which a key ultimate carcinogen, *N*-sulfonoxy-2-FAA, is formed in livers of rats susceptible to hepatocarcinogenesis by systemically administered 2-FAA and *N*-OH-2-FAA (*29–31*) could not account for mammary tumor induction by these compounds. The electrophilic arylamidonium ion could also arise from *N*-AcO-2-FAA which may be formed nonenzymatically with acetyl-CoA as the acetyl donor (*32*). Recently, we (*15*) reported that *N*-OH-2-FAA and *N*-OH-3-FAA were acetylated with acetyl-CoA at physiologic pH (the 3-isomer was acetylated to a greater extent). This suggested that the acetate esters might also be formed nonenzymatically in vivo. Accordingly, we compared the carcinogenicities of the acetate esters with that of *N*-fluorenylacetohydroxamic acids for the mammary gland after topical application (*15*). Whereas *N*-AcO-3-FAA showed a similar tumorigenicity to *N*-OH-3-FAA, *N*-AcO-2-FAA was much less carcinogenic (perhaps due to its decomposition at the site of application) than *N*-OH-2-FAA. In aqueous media, *N*-AcO-2-FAA decomposes to *N*-(3-OH)-2-FAA and *N*-(4-OH)-2-FAA (*33, 34*). In contrast, *N*-AcO-3-FAA is stable in buffers at physiologic pH with a half-life of approximately 24 hours (*15*). The differences in tumorigenicity between the 2 isomeric acetate esters may also be due to the differences in their electrophilic reactivities. In contrast to *N*-AcO-2-FAA, *N*-AcO-3-FAA did not form products indicating reaction of nucleophilic acceptors with the arylamidonium ion (*35, 36*). However, it was capable of modification of nucleophilic sites in proteins as shown by acetylation of the ε-amino group of lysine in the model protein, RNase (*37*). Although the same type of modification has been shown with *N*-AcO-2-FAA (*38*), acetylation of RNase by N-AcO-3-FAA was more extensive. Thus the modification of cellular nucleophiles through acetylation could be an alternative mechanism to explain the activity of *N*-AcO-3-FAA (*37*). Histologic evaluation of mammary gland tumors induced with *N*-AcO-3-FAA and *N*-OH-3-FAA by topical application shows that these compounds induced predominantly benign lesions (fibroadenomas and adenomas) in marked contrast to *N*-OH-2-FAA, which induced predominantly adenocarcinomas (table 3). A higher ratio of malignant to benign mammary gland tumors was also obtained with *N*-OH-2-FAA than with *N*-OH-3-FAA after systemic administration of the compounds (*12*). These data tempt one to speculate that the tumor type in the mammary gland may be determined

TABLE 3.—*Ratios of malignant to benign mammary gland tumors induced with* N-*OH-2-FAA,* N-*OH-3-FAA, and* N-*AcO-3-FAA*

Compound administered	Route of administration[a]	No. of rats with tumors/ No. of rats used [b]	Average No. of tumors/rat	Ratio
N-OH-2-FAA	ip	37/39	3.0	2.7
N-OH-3-FAA	ip	23/23	2.2	1.4
N-OH-2-FAA	Topical	21/31	1.5	2.1
N-OH-3-FAA	Topical	24/30	2.7	0.29
N-AcO-3-FAA	Topical	18/18	2.0	0.10

[a] The average total dose of compound was 0.17 and 0.02 mmol/rat by the ip and topical routes, respectively.

[b] The groups of rats used in this laboratory for carcinogenicity tests were combined to give these values [(*12, 15*); table 1].

by the nature of the modification of cellular macromolecules.

In tests recently reported, *N*-OH-2-FAA, which lacks electrophilic reactivity, was strongly carcinogenic at the site of sc injections, whereas the carcinogenity of its esters decreased with their increasing electrophilic reactivity (*39*). These results were interpreted as support for the hypothesis that the electrophile must be generated intracellularly to be most effective as a carcinogen. Accordingly, *N*-OH-2-FAA may be acetylated nonenzymatically in the cell to *N*-AcO-2-FAA, which could then be a potential source of the electrophilic arylamidonium ion at the site of sc injections or in the mammary gland. However, the yield of the acetate ester formed in the nonenzymatic reaction was low (*15*) as were the amounts of product indicative of arylamidonium ion formation after incubation of mammary gland homogenates with *N*-OH-2-FAA (*14*); this finding suggested that *N*-AcO-2-FAA formation alone could not account for the extensive modification of cellular macromolecules probably required for tumorigenesis.

Enzymatically generated *N*-acetoxyarylamines from *N*-arylacetohydroxamic acids have recently been detected as sources of electrophilic reactants in the rat mammary gland (*40, 41*). Bartsch et al. (*40, 42*) presented the first evidence that an enzyme (transcetylase) capable of transfer of the N-acetyl group from *N*-arylacetohydroxamic acid (donor) to the O-atom of the *N*-arylhydroxylamine (acceptor) is localized in the soluble fractions of rat liver and other tissues including the mammary gland of pregnant rats. Mammary gland transacetylase used *N*-OH-4-biphenylamine but not *N*-OH-2-FA as the acceptor of the acetyl group (*40*). This was disconcerting because both donors, *N*-OH-4-acetylaminobiphenyl and *N*-OH-2-FAA, are mammary gland carcinogens (*9, 10*). In later studies, King (*43*) referred to this enzyme as "arylhydroxamic acid *N,O*-acyltransferase" and found that in the mammary gland of lactating rats the enzyme activated both *N*-OH-4-acetylaminobiphenyl and *N*-OH-2-FAA to *N*-acetoxyarylamines which yielded adducts with tRNA (*41*). In addition, the ribosomal RNA adducts recovered from mammary gland after systemic administration of *N*-OH-2-FAA were consistent with an acyltransferase mechanism of activation (*41*). The recovery of large amounts of 2-FA from macromolecular adducts after incubation of unfortified mammary gland homogenates with *N*-OH-2-FAA in our earlier experiments (*14*) could also result from this mechanism.

The activation of *N*-arylacetohydroxamic acids to *N*-acetoxyarylamines does not occur exclusively in the mammary gland. Tissues not normally susceptible to tumor induction are capable of the same type of activation (*40, 43*). Consequently, whether an acyltransferase mechanism of activation may be implicated in mammary gland tumorigenesis awaits further investigation. At present, its involvement is proposed solely on the basis of the carcinogenicities of topically applied N-formyl, N-acetyl, and N-propionyl derivatives of *N*-OH-2-FAA that apparently related to the ability of acyltransferase to catalyze the formation of nucleic acid adducts from these hydroxamic acids (*44*).

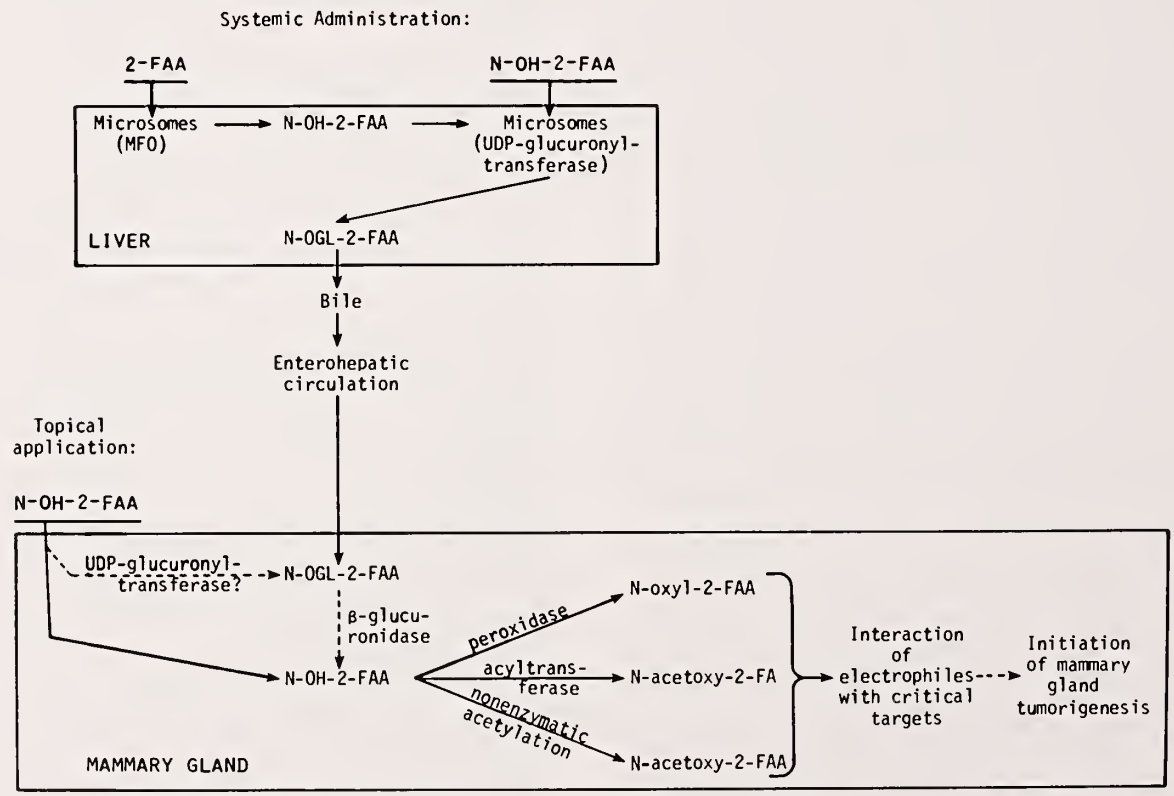
A recent report on peroxidase-catalyzed oxidation of *N*-OH-2-FAA in the presence of peroxide to a nitroxyl free radical in the rat mammary gland (*45*) revived interest in this potential mechanism of activation of *N*-arylacetohydroxamic acids. Previous studies were of the radical (*N*-oxyl-2-FAA) generated in vitro where it was found to be an unstable intermediate that undergoes dismutation in organic or aqueous solution to *N*-AcO-2-FAA and 2-nitrosofluorene (*46–49*). Thus through this mechanism the potentially electrophilic reactant *N*-AcO-2-FAA could be delivered intracellularly, where it might be an effective carcinogen. The other dismutation product, 2-nitrosofluorene, lacks electrophilic reactivity and is only weakly carcinogenic for the mammary gland after topical application (*50*); however, although it reacts with rat liver microsomal membranes to produce a nitroxyl free radical (*51*), we do not know whether this membrane-bound radical is active in carcinogenesis. There is also a possibility that a weakly electrophilic *N*-oxyl-2-FAA could react directly with nucleophilic sites on target macromolecules, but this idea has not been further explored (*46*). At present, support for a free radical mechanism in tumorigenesis comes indirectly from work with antioxidants (*52*). In general, antioxidants do protect animals against chemically induced cancer. However, the protection cannot always be attributed to the scavenging effect of antioxidants on the free radicals (*22*), as exemplified by the effect of disulfiram on 2-FAA-induced mammary gland carcinogenesis (*23*).

On the basis of the evidence currently available and reviewed in this report, the sequence of metabolic events in text-figure 3 may be considered as a possible mechanism underlying mammary tumorigenesis by *N*-arylacetamides and *N*-arylacetohydroxamic acids. Thus systemically administered 2-FAA undergoes activation in liver microsomes (mixed function oxidase) to *N*-OH-2-FAA. The proximate metabolite is then conjugated to *N*-OGl-2-FAA in the reaction involving UDP-glucuronic acid and microsomal glucuronyl transferase (*53*). The same conjugation occurs after systemic administration of *N*-OH-2-FAA to the rat. That *N*-OGl-2-FAA plays a role in mammary tumorigenesis of systemically administered 2-FAA and *N*-OH-2-FAA is a distinct possibility because its formation

in vivo is a major metabolic reaction, and the biliary excretion of this metabolite in the rat is much greater than that in the urine (*54, 55*). After excretion in the bile, most of the glucuronide conjugate appears to be hydrolyzed by β-glucuronidase of the bacteria in the cecum to *N*-OH-2-FAA and then enzymatically reduced to 2-FAA which is excreted with the feces (*56–58*). However, these metabolites and *N*-OGl-2-FAA could also be reabsorbed from the cecum into an enterohepatic circulation and could then reach the mammary gland. Radioactivity of labeled 2-FAA and *N*-OH-2-FAA given ip or orally to female rats was detected in their mammary glands (*59*). The isomeric *N*-OH-3-FAA, a systemic mammary gland carcinogen (*12*), may also form an *O*-glucuronide, but this conjugation has not been reported. It is possible that *O*-glucuronides of *N*-fluorenylacetohydroxamic acids are hydrolyzed by the mammary gland β-glucuronidase (*60*) to their aglycones. These aglycones would then be activated by the same mechanisms as those proposed for activation of *N*-fluorenylacetohydroxamic acids applied topically: one-electron oxidation to nitroxyl radicals catalyzed by peroxidase (*45*), formation of *N*-acetoxyarylamines catalyzed by acyltransferase (*41*), and to a minor extent the nonenzymatic acetylation (*15*). The latter activation seems more plausible for *N*-OH-3-FAA than for *N*-OH-2-FAA for the reasons discussed earlier. Alternatively, *N*-fluorenylacetohydroxamic acids applied topically might be conjugated in the mammary gland to reactive *O*-glucuronides. The reactivity of *N*-OGl-2-FAA with nucleic acids and homopolynucleotides in vitro is well established (*53, 61*), and its injection sc results in tumor induction at various sites including the mammary gland (*62*).

Because of scarcity of mammary parenchyma in young adult rats, much of the work concerning activation of *N*-arylacetohydroxamic acids to potential ultimate mammary gland carcinogens was done with mammary gland preparations from pregnant and lactating rats. Our data on the first step of activation of 2-FAA, i.e., its N-hydroxylation to *N*-OH-2-FAA, indicate that mammary gland of young adult female rats susceptible to carcinogenic action of this compound lacks capacity for N-hydroxylation of 2-FAA, which can be detected and induced in the mammary gland of lactating rats. Similarly, other activation reactions may be associated only with a certain stage of mammary gland development. Hormonal regulation of metabolic reactions cannot be ignored, especially when it is established that ovariectomized rats are not susceptible to mammary gland tumorigenesis induced chemically (*2, 15, 63*).

Studies on the development of mammary gland tumors are further complicated by the heterogeneous cellular composition of this tissue. Different epithelial and nonepithelial cell types may metabolize (activate) carcinogens in various ways that may result in the initiation of many types of neoplastic lesions (*64*). Our recent studies with cultures of mammary adenocarcinoma induced with *N*-OH-2-FAA suggest that this carcinogen transformed epithelial and nonepithelial cells of the rat mammary gland in vivo. Inoculations of cloned epithelial- and fibroblast-like cells



TEXT-FIGURE 3.—Possible mechanism of mammary gland tumorigenesis by 2-FAA and *N*-OH-2-FAA; ——→ = events supported by experimental evidence; —·—→ = proposed events.

from this tumor produced adenocarcinomas and fibrosarcomas, respectively, in isologous hosts (*65*). Future endeavors in mammary gland tumorigenesis should rely on homogeneous cell culture systems for studies of carcinogen-initiated critical cellular events.

ADDENDUM

Since this paper was submitted, we found NADPH-dependent cytochrome c reductase activity in the mammary gland microsomes using 0.3 M potassium phosphate buffer, pH 7.4, for the enzyme assay (*66*). The enzyme activity, 15 nmol cytochrome c reduced/mg protein/min, was within the range reported by other investigators (*19, 67*).

REFERENCES

(*1*) Huggins C, Briziarelli G, Sutton H Jr: Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. J Exp Med 109:25–41, 1959

(*2*) Huggins C, Grand LC, Brillantes FP: Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. Nature 189:204–207, 1961

(*3*) Arcos JC, Argus MF, eds: Structure–activity relationships. *In* Chemical Induction of Cancer. Structural Bases and Biological Mechanism, vol. IIB. New York: Academic Press, 1974, pp 30–31

(*4*) Griswold DP Jr, Casey AE, Weisburger EK, et al: On the carcinogenicity of a single intragastric dose of hydrocarbons, nitrosamines, aromatic amines, dyes, coumarins, and miscellaneous chemicals in female Sprague-Dawley rats. Cancer Res 26:619–626, 1966

(*5*) Dunning WF, Curtis MR, Madsen ME: The induction of neoplasms in five strains of rats with acetylaminofluorene. Cancer Res 7:134–140, 1947

(*6*) Gullino PM, Pettigrew HM, Grantham FM: *N*-Nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54:401–414, 1975

(*7*) Miller EC, Miller JA: The metabolism of chemical carcinogens to reactive electrophiles and their possible mechanisms of action in carcinogenesis. *In* Chemical Carcinogens (Searle CE, ed), American Cemical Society Monograph No. 173. Washington, D.C.: Am Chem Soc, 1976, pp 737–762

(*8*) Cramer JW, Miller JA, Miller EC: N-Hydroxylation: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J Biol Chem 235:885–888, 1960

(*9*) Miller EC, Miller JA, Hartmann HA: N-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res 21:815–824, 1961

(*10*) Miller JA, Wyatt CS, Miller EC: The N-hydroxylation of 4-acetylaminobiphenyl by the rat and dog and the strong carcinogenicity of *N*-hydroxy-4-acetylaminobiphenyl in the rat. Cancer Res 21:1465–1473, 1961

(*11*) Gutmann HR, Galitski SB, Foley W: The conversion of noncarcinogenic aromatic amides to carcinogenic arylhydroxamic acids by synthetic N-hydroxylation. Cancer Res 27:1443–1455, 1967

(*12*) Gutmann HR, Leaf DS, Yost Y, et al: Structure–activity relationships of *N*-acylhydroxylamines in the rat. Cancer Res 30:1485–1498, 1970

(*13*) Russo J, Russo IH: DNA labeling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. J Natl Cancer Inst 61:1451–1459, 1978

(*14*) Malejka-Giganti D, Gutmann HR, Rydell RE: Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids. Cancer Res 33:2489–2497, 1973

(*15*) Malejka-Giganti D, Rydell RE, Gutmann HR: Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids and their acetates. Cancer Res 37:111–117, 1977

(*16*) Lotlikar PD, Zaleski K: Ring and N-hydroxylation of 2-acetamidofluorene by rat liver reconstituted cytochrome P_{450} enzyme system. Biochem J 150:561–564, 1975

(*17*) Gutmann HR, Bell P: N-Hydroxylation of arylamides by the rat and guinea pig. Evidence for substrate specificity and participation of cytochrome $P_{1\text{-}450}$. Biochim Biophys Acta 498:229–243, 1977

(*18*) Malejka-Giganti D, McIver RC, Glasebrook AL, et al: Induction of microsomal N-hydroxylation of *N*-2-fluorenylacetamide in rat liver. Biochem Pharmacol 27:61–69, 1978

(*19*) Bruder G, Fink A, Jarasch ED: The b-type cytochrome in endoplasmic reticulum of mammary gland epithelium and milk fat globule membranes consists of two components, cytochrome b_5 and cytochrome P_{420}. Exp Cell Res 117:207–217, 1978

(*20*) Omura T, Sato R: The carbon monoxide-binding pigment of liver microsomes. J Biol Chem 239:2370–2378, 1964

(*21*) Falk JE: Haems. *In* Porphyrins and Metalloporphyrins, vol 2. New York: Elsevier, 1964, pp 181–182

(*22*) Wattenberg LW: Inhibition of chemical carcinogenesis. J Natl Cancer Inst 60:11–18, 1978

(*23*) Malejka-Giganti D, McIver RC, Rydell RE: Inhibitory effect of disulfiram on mammary tumor induction by *N*-2-fluorenylacetamide and on its metabolic conversion to *N*-hydroxy-*N*-2-fluorenylacetamide. JNCI 64:1471–1477, 1980

(*24*) Marselos M, Alakuijala P, Lang M, et al: Studies on the mechanism by which disulfiram and diethyldithiocarbamate affect drug metabolism. *In* Microsomes and Drug Oxidations (Ullrich V, Roots I, Hildebrandt A, et al, eds). New York: Pergamon Press, 1977, pp 589–596

(*25*) King CM, Gutmann HR, Chang SF: The oxidation of *o*-aminophenols by cytochrome c and cytochrome oxidase. IV. Interaction of 2-imino-1,2-fluorenoquinone and of 2-imino-2,3-fluorenoquinone with bovine serum albumin. J Biol Chem 238:2199–2205, 1963

(*26*) Grantham PH, Weisburger EK, Weisburger JH: Dehydroxylation and deacetylation of *N*-hydroxy-*N*-2-fluorenylacetamide by rat liver and brain homogenates. Biochim Biophys Acta 107:414–424, 1965

(*27*) Miller JA: Carcinogenesis by chemicals: An Overview. GHA Clowes Memorial Lecture. Cancer Res 30:559–576, 1970

(*28*) Irving CC, Janss DH, Russell LT: Lack of *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and Zymbal's gland of the rat. Cancer Res 31:387–391, 1971

(*29*) Debaun JR, Miller EC, Miller JA: *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein (methion-*S*-yl) binding in rat liver. Cancer Res 30:577–595, 1970

(*30*) Gutmann HR, Malejka-Giganti D, Barry EJ, et al: On the correlation between the hepatocarcinogenicity of the carcinogen, *N*-2-fluorenylacetamide, and its metabolic activation by the rat. Cancer Res 32:1554–1561, 1972

(31) WEISBURGER JH, YAMAMOTO RS, WILLIAMS GM, et al: On the sulfate ester of *N*-hydroxy-*N*-2-fluorenylacetamide as a key ultimate hepatocarcinogen in the rat. Cancer Res 32:491–500, 1972

(32) LOTLIKAR PD, LUHA L: Acetylation of the carcinogen *N*-hydroxy-2-acetylaminofluorene by acetyl coenzyme-A to form a reactive ester. Mol Pharmacol 7:381–388, 1971

(33) SCRIBNER JD, MILLER JA, MILLER EC: Nucleophilic substitution on carcinogenic *N*-acetoxy-*N*-arylacetamides. Cancer Res 30:1570–1579, 1970

(34) SCRIBNER JD: Conversion of the carcinogen *N*-acetoxy-2-acetamidofluorene to 4-hydroxy-2-acetamidofluorene. J Am Chem Soc 99:7383–7384, 1977

(35) ZIEVE FJ, GUTMANN HR: Reactivities of the carcinogens, *N*-hydroxy-2-fluorenylacetamide and *N*-hydroxy-3-fluorenylacetamide, with tissue nucleophiles. Cancer Res 31:471–476, 1971

(36) YOST Y, GUTMANN HR, RYDELL RE: The carcinogenicity of fluorenylhydroxamic acids and *N*-acetoxy-*N*-fluorenylacetamides for the rat as related to the reactivity of the esters toward nucleophiles. Cancer Res 35:447–459, 1975

(37) BARRY EJ, GUTMANN HR: Protein modifications by activated carcinogens. II. The acetylation of ribonuclease by *N*-acetoxy-3-fluorenylacetamide. Chem Biol Interact 13: 47–55, 1976

(38) BARRY EJ, GUTMANN HR: Protein modifications by activated carcinogens. I. The acetylation of ribonuclease by *N*-acetoxy-2-fluorenylacetamide. J Biol Chem 248:2730–2731, 1973

(39) BARTSCH H, MALAVEILLE C, STICH HF, et al: Comparative electrophilicity, mutagenicity, DNA repair induction activity, and carcinogenicity of some *N*- and *O*-acyl derivatives of *N*-hydroxy-2-aminofluorene. Cancer Res 37: 1461–1467, 1977

(40) BARTSCH H, DWORKIN C, MILLER EC, et al: Formation of electrophilic *N*-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-4-acetylaminobiphenyl. Biochim Biophys Acta 304:42–55, 1973

(41) KING CM, TRAUB NR, LORTZ ZM, et al: Metabolic activation of arylhydroxamic acids by *N*-*O*-acyltransferase of rat mammary gland. Cancer Res 39:3369–3372, 1979

(42) BARTSCH H, DWORKIN M, MILLER JA, et al: Electrophilic *N*-acetoxyaminoarenes derived from carcinogenic *N*-hydroxy-*N*-acetylaminoarenes by enzymatic deacetylation and transacetylation in liver. Biochim Biophys Acta 286:272–298, 1972

(43) KING CM: Mechanism of reaction, tissue distribution, and inhibition of arylhydroxamine acid acyltransferase. Cancer Res 34:1503–1515, 1974

(44) KING CM, ALLABEN WT: The role of arylhydroxamic acid *N*-*O*-acyltransferase in the carcinogenicity of aromatic amines. *In* Conjugation Reactions in Drug Biotransformation (Aitio A, ed). New York: Elsevier/North Holland, 1978, pp 431–441

(45) REIGH DL, STUART M, FLOYD RA: Activation of the carcinogen *N*-hydroxy-2-acetylaminofluorene by rat mammary peroxidase. Experientia 34:107–108, 1978

(46) BARTSCH H, TRAUT M, HECKER E: On the metabolic activation of *N*-hydroxy-*N*-2-acetylaminofluorene. II. Simultaneous formation of 2-nitrosofluorene and *N*-acetoxy-*N*-2-acetylaminofluorene from *N*-hydroxy-*N*-2-acetylaminofluorene via a free radical intermediate. Biochim Biophys Acta 237:556–566, 1971

(47) BARTSCH H, HECKER E: On the metabolic activation of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene. III. Oxidation with horseradish peroxidase to yield 2-nitrosofluorene and *N*-acetoxy-*N*-2-acetylaminofluorene. Biochim Biophys Acta 237:567–578, 1971

(48) FLOYD RA, SOONG LM, CULVER PL: Horseradish peroxidase/hydrogen peroxide-catalyzed oxidation of the carcinogen *N*-hydroxy-*N*-acetyl-2-aminofluorene as effected by cyanide and ascorbate. Cancer Res 36:1510–1519, 1976

(49) FLOYD RA, SOONG LM, WALKER RN: Lipid hydroperoxide activation of *N*-hydroxy-*N*-acetylaminofluorene via a free radical route. Cancer Res 36:2761–2767, 1976

(50) MALEJKA-GIGANTI D, GUTMANN HR: *N*-Hydroxy-2-fluorenylacetamide, an active intermediate of the mammary carcinogen *N*-hydroxy-2-fluorenylbenzenesulfonamide. Proc Soc Exp Biol Med 150:92–97, 1975

(51) FLOYD RA, SOONG LM, STUART MA, et al: Free radicals and carcinogenesis. Some properties of the nitroxyl free radicals produced by covalent binding of 2-nitrosofluorene to unsaturated lipids of membranes. Arch Biochem Biophys 185:450–457, 1978

(52) PRYOR WA: The formation of free radicals and the consequences of their reactions in vivo. Photochem Photobiol 28:787–801, 1978

(53) IRVING CC: Metabolic activation of N-hydroxy compounds by conjugation. Xenobiotica 1:387–398, 1971

(54) IRVING CC, WISEMAN R JR, HILL JT: Biliary excretion of the *O*-glucuronide of *N*-hydroxy-2-acetylaminofluorene by the rat and rabbit. Cancer Res 27:2309–2317, 1967

(55) IRVING CC: Conjugates of N-hydroxy compounds. *In* Metabolic Conjugation and Metabolic Hydrolysis (Fishman WH, ed), vol 1. New York: Academic Press, 1970, pp 53–119

(56) WEISBURGER JH, GRANTHAM PH, HORTON RE, et al: Metabolism of the carcinogen *N*-hydroxy-*N*-2-fluorenylacetamide in germ-free rats. Biochem Pharmacol 19: 151–162, 1970

(57) GRANTHAM PH, HORTON RE, WEISBURGER EK, et al: Metabolism of the carcinogen *N*-2-fluorenylacetamide in germfree and conventional rats. Biochem Pharmacol 19:163–171, 1970

(58) WILLIAMS JR JR, GRANTHAM PH, MARSH HH III, et al: Participation of liver fractions and of intestinal bacteria in the metabolism of *N*-hydroxy-*N*-2-fluorenylacetamide in the rat. Biochem Pharmacol 19:173–188, 1970

(59) JANSS DH, IRVING CC: Radioactivity in rat mammary gland after the administration of 2-acetylaminofluorene-9-^{14}C and its *N*-hydroxy metabolite. J Natl Cancer Inst 49:765–771, 1972

(60) KNOBIL E: The relation of some steroid hormones to β-glucuronidase activity. Endocrinology 50:16–28, 1952

(61) IRVING CC: Influence of the aryl group on the reaction of glucuronides of *N*-arylacetylhydroxamic acids with polynucleotides. Cancer Res 37: 524–528, 1977

(62) IRVING CC, WISEMAN R JR: Studies on the carcinogenicity of the glucuronides of *N*-hydroxy-2-acetylaminofluorene and *N*-2-fluorenylhydroxylamine in the rat. Cancer Res 31:1645–1648, 1971

(63) DAO TL: Studies on mechanism of carcinogenesis in the mammary gland. Prog Exp Tumor Res 11:235–261, 1969

(64) SLEMMER G: Interactions of separate types of cells during normal and neoplastic mammary gland growth. J Invest Dermatol 63:27–47, 1974

(65) MALEJKA-GIGANTI D, POTTER AH, RYDELL RE: A fibrosarcoma component in culture of a chemically induced rat mammary adenocarcinoma. Lab Invest 42:627–635, 1980

(66) PHILLIPS AH, LANGDON RG: Hepatic triphosphopyridine

nucleotide-cytochrome *c* reductase: Isolation, characterization, and kinetic studies. J Biol Chem 237:2652–2660, 1962

(*67*) Rikans LE, Gibson DD, McCay PB: Evidence for the presence of cytochrome P-450 in rat mammary gland. Biochem Pharmacol 28:3039–3042, 1979

The Metabolic Basis for Inhibitory Effects in Chemical Carcinogenesis by Arylamines[1]

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ABSTRACT—The inhibition of the carcinogenicity of *N*-2-fluorenylacetamide (2-FAA) by acetanilide (AA), *p*-hydroxyacetanilide (*p*-OH-AA), butylated hydroxytoluene (BHT), and chloramphenicol is reviewed. The mechanisms of action by which inhibition may occur are as follows: 1) inhibition of the binding of the activated metabolite of FAA to cellular macromolecules (DNA, RNA, and proteins), 2) changes in the amount of the N-hydroxylated metabolite, believed to be the first step of activation of FAA, formed and excreted in the urine, 3) induction of the enzyme glucuronyl transferase which increases the formation of glucosiduronic acid that results in a rapid excretion of the carcinogens, 4) depletion of sulfate by *p*-OH-AA, the major metabolite of AA. The sulfate ion is required for the second activation step, i.e., the formation of the sulfate ester of *N*-OH-FAA. The data show several of the inhibitors may operate by one or more of the above mechanisms of inhibition.— Natl Cancer Inst Monogr 58: 79–84, 1981.

The carcinogenicity of agents of the arylamine type, such as 2-FAA, depends on a number of endogenous and exogenous factors (*1, 2*). Thus the incidence and type of tumors, the latent period, and the organ affected depend on the species, strain and sex of the test animals, as well as dietary and other factors.

Numerous investigations have been conducted on the inhibition of the carcinogenicity of one chemical by the administration of related noncarcinogenic analogs. However, the underlying mechanisms have not always been clearly delineated.

Chemical compounds that have inhibited the carcinogenicity of 2-FAA and its derivatives are: chloramphenicol (*3*), 3-methylcholanthrene (*4*), phenobarbital (*5*), AA (*6, 7*), *p*-OH-AA (*8*), and BHT (*9*). This paper is a survey of the studies from our laboratory on the metabolic alterations of 2-FAA or *N*-OH-2-FAA as a result of pretreatment of animals with several of the above-named inhibitors.

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; AA = acetanilide; *p*-OH-AA = *p*-hydroxy-AA; BHT = butylated hydroxytoluene; *N*-OH-2-FAA = *N*-hydroxy-2-FAA; FDAA = *N*-2-fluorenyldiacetamide.

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Among several plausible mechanisms by which inhibition of 2-FAA-induced carcinogenesis might occur, the following are discussed: 1) inhibition of binding to cellular macromolecules, 2) changes in the amount of the activated metabolite *N*-OH-2-FAA formed and excreted in urine, 3) increase in the rate of glucosiduronic acid conjugation and excretion, 4) depletion of sulfate by *p*-OH-AA.

INHIBITION OF BINDING TO CELLULAR MACROMOLECULES

If the binding of carcinogens to cellular receptors was the initial event responsible for the malignant transformation of cells, then the carcinogenicity should be decreased by the administration of compounds which compete for the receptor sites.

Acetanilide

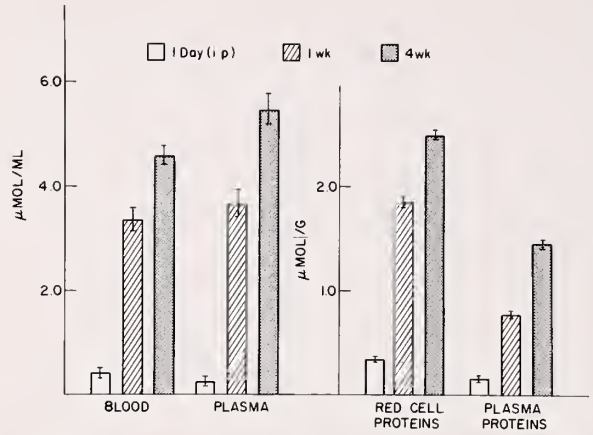
The binding to cellular receptors of AA, a noncarcinogen, did occur after either a single ip dose or 1 and 4 weeks of continuous feeding (text-fig. 1) (*10*). Binding to the protein fraction of blood and plasma was low 24 hours after injection. However, the activity increased significantly at 1 and 4 weeks. A comparison of binding to the cellular macromolecules of liver (text-fig. 2) showed that the radioactivity bound to soluble RNA and protein reached limiting values soon after 1 week.

The activity bound to microsomal RNA and protein at 1 week was only 71 and 76%, respectively, of the 4-week values. The greatest difference was seen in the DNA fraction, in which the level of binding at 1 week was only 17% of that at 4 weeks. These data showed that the inhibitor AA did indeed bind to macromolecules.

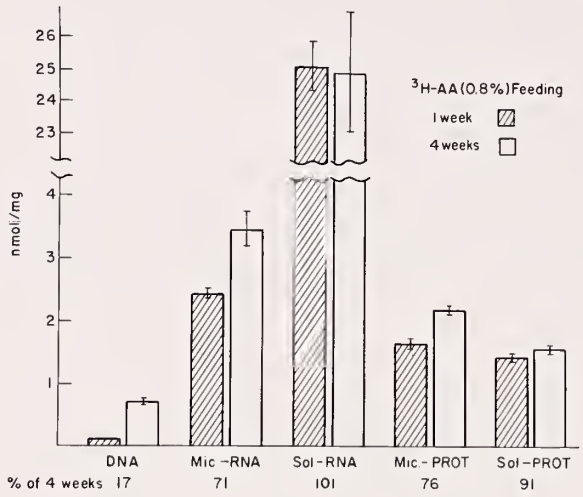
The distribution of isotope in the liver and protein, illustrated in text-figure 3, was determined in groups of rats fed a control diet, AA, AA plus 2-FAA, and 2-FAA (*11*) before they received a dose of labeled 2-FAA. The controls had the largest isotope content in the liver after a single dose of [^{14}C]2-FAA, whereas the rats prefed unlabeled FAA had the lowest amount. The AA and AA plus FAA groups, which showed intermediate values, demonstrated that AA binds to certain receptors and thus decreases the level of 2-FAA metabolites bound.

Butylated Hydroxytoluene

Ulland et al. (*9*) found that feeding BHT with 2-FAA or *N*-OH-2-FAA reduced the incidence of liver or mammary



TEXT-FIGURE 1.—Distribution of [^{3}H]AA in blood plasma and protein fractions (*10*).

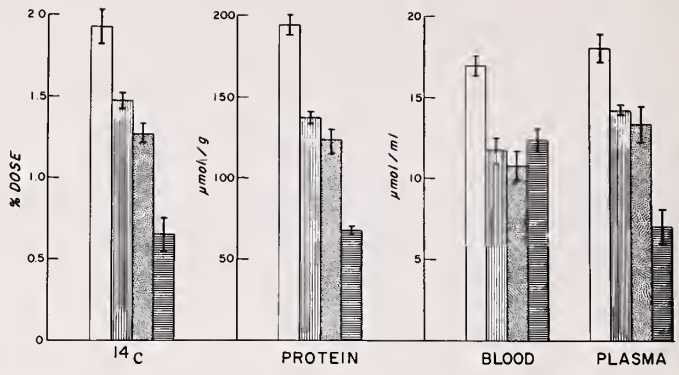


TEXT-FIGURE 2.—Binding of AA metabolites to liver macromolecules after 1 and 4 wk of feeding [^{3}H]AA at 59 mmol/kg in the diet (*10*). Mic = microsomal; Sol = soluble; Prot = protein.

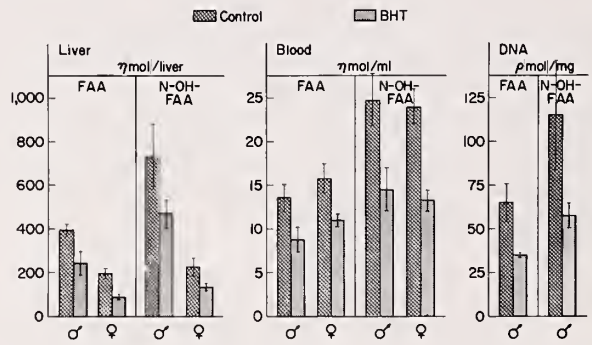
cancers. In our (*12*) metabolic study, we showed that animals prefed with BHT had a reduction in the total amount of radioactivity in the liver (text-fig. 4), but more isotope was in the livers of male than female rats. The binding to DNA paralleled the values in the whole liver. With both 2-FAA and *N*-OH-2-FAA, BHT reduced the amount of carcinogen bound to DNA, but more of the isotope was bound to the DNA of animals given injections of *N*-OH-2-FAA than those given 2-FAA.

Chloramphenicol

It was obvious that prefeeding with chloramphenicol (*13*) led to considerable binding on cellular proteins because the levels of radioactivity in protein from liver, plasma, and red cells after the administration of labeled carcinogen were, respectively, 32, 58, and 15% of control



TEXT-FIGURE 3.—Distribution of radioactivity in the liver, plasma, and blood of controls and rats treated with 59 mmol AA/kg, 59 mmol AA/kg plus 0.89 mmol 2-FAA/kg, and 0.89 mmol 2-FAA/kg. Injections of [^{14}C]2-FAA were given 24 hr prior to autopsy (*11*). *Open bars* = control; *vertical-hatched bars* = AA; *dotted bars* = AA + 2-FAA; *horizontal-hatched bars* = 2-FAA.



TEXT-FIGURE 4.—Levels of radioactivity representing metabolites of 2-FAA and *N*-OH-2-FAA in the liver and blood of males and females and bound to liver DNA in males 48 hr after an ip injection of [^{14}C]-labeled carcinogen (*12*).

values (table 1). Interestingly, chloramphenicol actually led to an increase in the amount of the activated *N*-OH-2-FAA excreted in the urine.

CHANGES IN THE AMOUNT OF *N*-HYDROXY-*N*-2-FLUORENYLACETAMIDE FORMED AND EXCRETED IN THE URINE

N-Hydroxylation of 2-FAA is generally accepted as the first step for activation that is necessary for tumor formation. Therefore, factors which influence this step could alter the carcinogenic response.

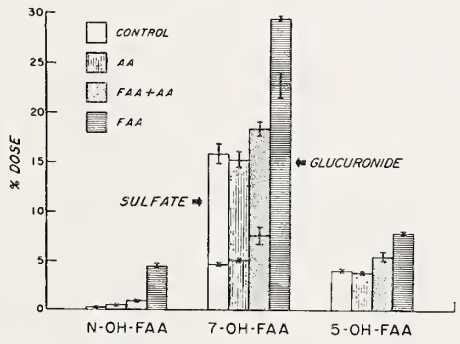
Acetanilide

Text-figure 5 shows that rats pretreated with 2-FAA excreted a considerably larger percentage of a dose as the important carcinogenic intermediate *N*-OH-2-FAA, whereas a group pretreated with AA alone or AA plus FAA showed a significant decrease in the level of the N-OH-derivative.

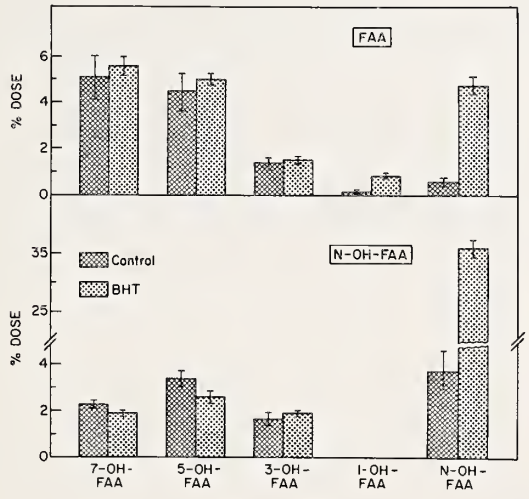
TABLE 1.—*Excretion of radioactivity, urinary metabolites, and total bound liver radioactivity in rats fed 2-FDAA and chloramphenicol for 6 wk, followed by an ip injection of labeled 2-FDAA*[a]

Group	Urine[b]				Feces[b]	Liver[b]	Proteins, μmol/g		
	Total	Free compound	Glucuro-nides	Sulfates			Liver	Plasma	Red cells
FDAA (I)	83	2.2	47	14	2.2	0.75	71	121	78
FDAA + chloramphenicol (II)	71	1.6	53	5.7	3.8	0.80	23	67	12
Ratio: (II/I) × 100 (percent)	86	73	113	41	173	107	32	58	15

[a] Data are from (*13*).
[b] Values indicate percentage of total dose excreted.



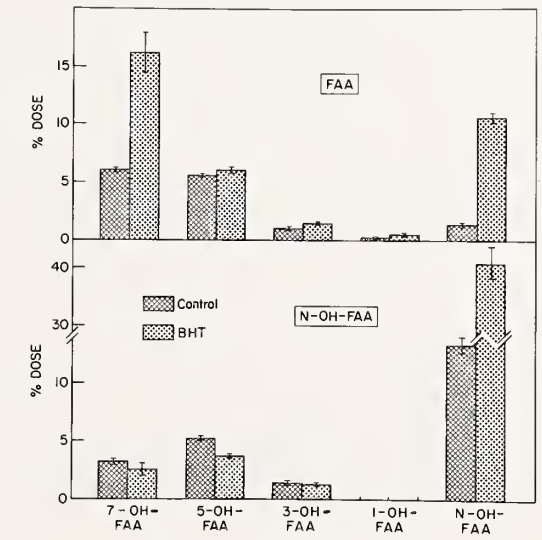
TEXT-FIGURE 5.—Major ether-soluble metabolites of 2-FAA in urine of control rats and rats fed 59 mmol AA/kg, 59 mmol AA + 0.89 mmol 2-FAA/kg, and 0.89 mmol 2-FAA/kg for 6 wk. Injections of [^{14}C]2-FAA were given 24 hr before autopsy (*11*).



TEXT-FIGURE 6.—Major metabolites of 2-FAA (*upper*) and *N*-OH-2-FAA (*lower*) in the glucosiduronic acid fraction of the urine of male rats on control and BHT (6,600 ppm) diets for 4 wk before ip injections of the labeled carcinogen (*12*).

Butylated Hydroxytoluene

The major metabolites of 2-FAA and *N*-OH-2-FAA in controls and BHT-treated rats are shown in text-figure 6.



TEXT-FIGURE 7.—Major metabolites of 2-FAA (*upper*) and *N*-OH-2-FAA (*lower*) in the glucosiduronic acid fraction of the urine of female rats on control and BHT (6,600 ppm) diets for 4 wk before ip injections of the labeled carcinogen (*12*).

Pretreatment of male rats with BHT had no significant effect on the ring-hydroxylated metabolites; however, a tremendous increase in the N-hydroxylated metabolite was observed. With *N*-OH-2-FAA after BHT pretreatment, amounts of the metabolites hydroxylated at the 7-, 5-, 3-, and 1-positions were lower, whereas a larger amount of the N-OH-derivative was detected. Pretreatment with BHT again failed to affect the production of ring-hydroxylated metabolites but appreciably increased the amount of N-OH-derivative.

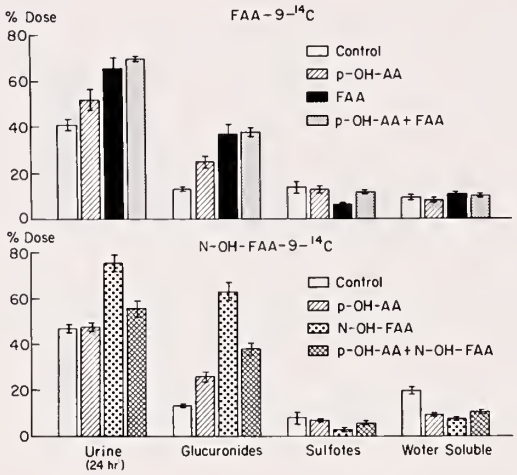
The situation was similar for female rats (text-fig. 7) administered 2-FAA, but the relative amounts of ring- and N-hydroxylated metabolites differed. Pretreatment with BHT showed an increase in the N-OH- as well as the 7-OH-derivatives. When *N*-OH-2-FAA was injected, pretreatment with BHT increased the excretion of N-OH-derivatives significantly. These data indicate that glucuronic acid rapidly conjugates the *N*-OH-2-FAA that reaches the liver and thus hastens its elimination from the animal.

INCREASED RATE OF GLUCURONIC ACID CONJUGATION AND EXCRETION

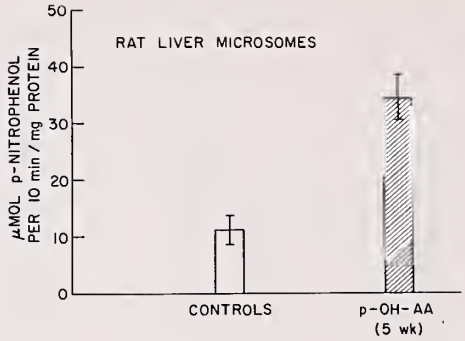
p-Hydroxyacetanilide

Text-figure 8 shows that pretreatment of rats with *p*-OH-AA, 2-FAA, and *p*-OH-AA plus FAA considerably increased the amount of isotope in the urine (*14*). The glucosiduronic acid fraction revealed even more pronounced differences. Treatment with *p*-OH-AA led to a twofold increase over the control value, whereas pretreatment with 2-FAA and *p*-OH-AA plus FAA almost tripled the control value. The differences in the glucuronide fraction paralleled those of the urinary excretion. In the *N*-OH-2-FAA study, the *p*-OH-AA group yielded a twofold increase over control values, but the groups given *p*-OH-AA and *N*-OH-2-FAA and the *N*-OH-2-FAA showed threefold and fivefold increases, respectively.

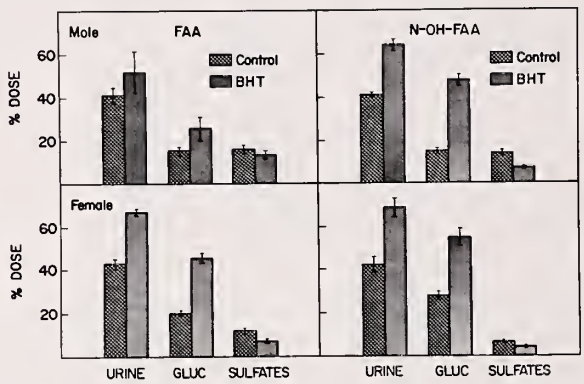
Determination of the effect of *p*-OH-AA treatment on the glucuronyl transferase levels of the liver revealed that the increase in the enzyme activity is threefold (text-fig. 9), which may account for the higher levels of glucuronide conjugation and subsequent excretion.



TEXT-FIGURE 8.—Excretion and fractionation of urinary radioactivity in control and pretreated rats (*14*).



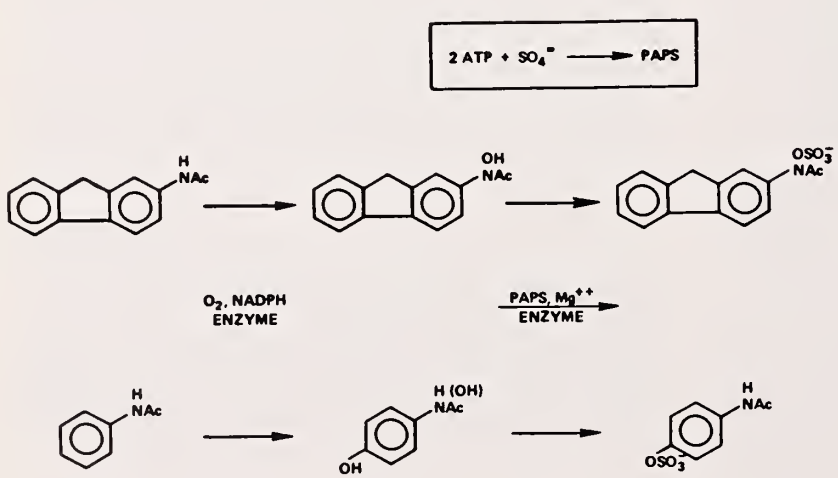
TEXT-FIGURE 9.—Glucuronyl transferase activity in control and *p*-OH-AA-treated rats (*14*).



TEXT-FIGURE 10.—Levels of radioactivity representing metabolites of 2-FAA (*left*) and *N*-OH-2-FAA (*right*) excreted in the urine in a 24-hr period after a single ip dose of labeled carcinogen administered to rats fed control and BHT (6,600 ppm) diets for the previous 4 wk (*12*). Gluc = glucosiduronic acid.

Butylated Hydroxytoluene

In rats of both sexes, BHT increased significantly the amount of radioactivity excreted in the urine from 2-FAA and *N*-OH-2-FAA (text-fig. 10). Thus pretreatment with BHT led to almost a twofold increase in the percent of



TEXT-FIGURE 11.—Possible mechanism of interference by AA metabolites with N-OH-amides (*15*). PAPS = 3-phosphoadenosine-5-phosphosulfate.

TABLE 2.—*Restoration by dietary sulfate of liver carcinogenesis in rats in the inhibited system AA + N-OH-2-FAA*

Experimental diets[a]	Final No. of rats	Final body weight, g	Liver weight		Liver histology						Rats with liver neoplasms, %
					Hyperplasia			Hepatoma			
			g	g/100 g	None	Area	Nodular	Focal in nodule	Small	Large	
N-OH-2-FAA	7[b]	262 ± 14[c]	26.2 ± 4.8	9.7				2		5	100
N-OH-2-FAA + AA	12	336 ± 9	11.4 ± 0.5	3.4	3	4	2	2			17
N-OH-2-FAA + AA + $SO_4^=$ [d]	13	337 ± 6	12.1 ± 0.4	3.6		1	9	1	1	1	23
N-OH-2-FAA + AA + $SO_4^=$ (threefold level)	14	334 ± 6	12.5 ± 0.4	3.7		2	4	5		3	57
N-OH-2-FAA + AA + $PO_4^{\equiv}$ [d]	12	314 ± 11	10.8 ± 0.4	3.4	4	3	4			1	8.3
N-OH-2-FAA + $SO_4^=$	5	247 ± 6	22.4 ± 2.0	9.1						5	100
N-OH-2-FAA + $PO_4^{\equiv}$	5	287 ± 9	23.8 ± 0.8	8.3						5	100
$SO_4^=$	6	361 ± 13	10.4 ± 0.4	2.9	6						0
$SO_4^=$ (threefold level)	5	379 ± 8	10.7 ± 0.2	2.8	5						0
$PO_4^{\equiv}$	6	363 ± 12	10.2 ± 0.4	2.8	6						0

[a] Data are from (*15*). Ingredients were added in the following concentrations; *N*-OH-2-FAA, 1.34 mmol/kg; AA, 59 mmol/kg; $NaSO_4$, 59 mmol/kg; $NaSO_4$ (threefold level), 178 mmol/kg; Na_2HPO_4, 158 mmol/kg.

[b] Four rats died after 4 wk on the diet; 5 others with enlarged hepatocellular carcinomas died after 24 wk in the experiment.

[c] Values are weights ± SE.

[d] $SO_4^=$ and $PO_4^{\equiv}$ = sulfate and phosphate ions, respectively.

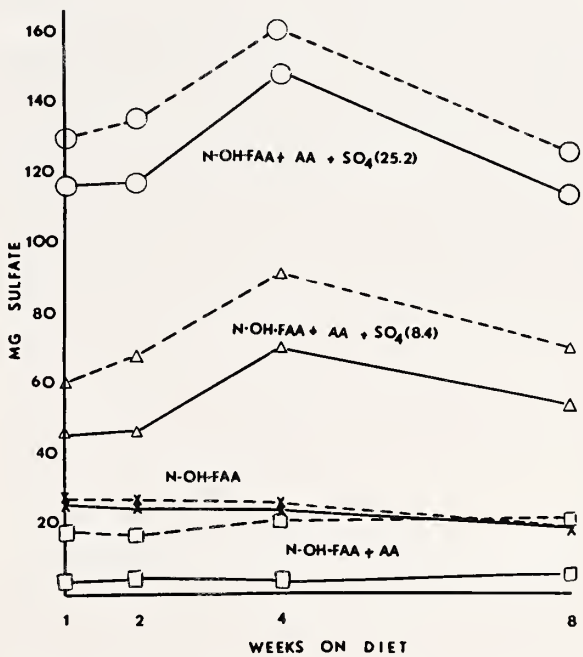
dose of 2-FAA excreted as glucuronides and almost threefold in the proportion of *N*-OH-2-FAA.

DEPLETION OF SULFATE BY *p*-HYDROXYACETANILIDE

Through its major metabolite, *p*-OH-AA, AA decreases the availability of sulfate necessary for liver carcinogenesis by acting as a sulfate trap (text-fig. 11).

The second activation step to yield the reactive form of the carcinogenic arylamine and arylamide is thought to be esterification of the N-OH-derivative. These derivatives of 2-FAA showed greater mutagenicity and reactivity with tissue nucleophiles than did the hydroxamic acids (*1*).

The involvement of sulfate as a factor is depicted in table 2. Although AA inhibited the effect of the carcinogen in the liver, the addition of excess sulfate again increased the number of rats with advanced lesions. An assay of urinary, free, and total sulfate (text-fig. 12) showed that groups of rats fed *N*-OH-2-FAA exhibited no change with respect to normal levels of sulfate present. When AA was added to the diet containing the carcinogen, levels of free sulfate were low. These data showed that trapping the sulfate usually involved in esterification and thus activation of the N-OH-derivatives led to a decrease in the carcinogenic effect.



TEXT-FIGURE 12.—Daily urinary excretion of free and total sulfate by rats on various regimens of *N*-OH-2-FAA or *N*-OH-2-FAA + AA or the latter plus 1 or 3 equivalents of dietary sodium sulfate ($SO_4^=$); _____ = free urinary sulfate; - - - = total urinary sulfate (*15*).

CONCLUSIONS

The current views on the metabolic activation of arylamines such as 2-FAA indicate that two steps are required for tumor induction. N-Oxidation of arylamines and arylamides is considered to be the initial activation step. The data presented showed that AA inhibits the usual progressively increased excretion of the N-OH-derivative in rats fed 2-FAA, perhaps by interference with hydroxylated systems acting on 2-FAA. The protective effects of *p*-OH-

AA and BHT may rest partly on the increased glucuronyl transferase activity that results in large glucuronic acid conjugation. The second step of activation, esterification of the N-OH-derivative, may be influenced by both a decrease in the amount of *N*-OH-2-FAA due to an increase in glucuronide conjugation and also the availability of sulfate, which is necessary for the formation of the reactive species.

Inhibitors, such as chloramphenicol, which have strong affinities for receptor sites, may prevent the interaction of a carcinogen with specific receptors that may be necessary to initiate the carcinogenic process.

REFERENCES

(*1*) Miller JA: Carcinogenesis by chemicals: An overview. Cancer Res 30:559–576, 1970

(*2*) Weisburger JH, Weisburger EK: Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(*3*) Puron R, Firminger HI: Protection against induced cirrhosis and hepatocellular carcinoma in rats by chloramphenicol. J Natl Cancer Inst 35:29–37, 1965

(*4*) Lotlikar PD, Enomoto M, Miller JA, et al: Species variations in N- and ring-hydroxylation of 2-acetylaminofluorene and effects on 3-methylcholanthrene pretreatment. Proc Soc Exp Biol Med 125:341–346, 1967

(*5*) Peraino C, Fry RJ, Staffeldt E: Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res 31: 1506–1512, 1971

(*6*) Yamamoto RS, Glass RM, Frankel HH, et al: Inhibition of the toxicity and carcinogenicity of *N*-2-fluorenylacetamide by acetanilide. Toxicol Appl Pharmacol 13: 108–117, 1968

(*7*) Yamamoto RS, Frankel HH, Weisburger JH: Effects of isomers of acetotoluidide and aminobenzoic acids on the toxicity and carcinogenicity of *N*-2-fluorenylacetamide. Toxicol Appl Pharmacol 17:98–106, 1970

(*8*) Weisburger JH, Weisburger EK, Madison RM, et al: Effect of acetanilide and *p*-hydroxyacetanilide on the carcinogenicity of *N*-2-fluorenylacetamide and *N*-hydroxy-*N*-2-fluorenylacetamide in mice, hamsters, and female rats. J Natl Cancer Inst 51:235–240, 1973

(*9*) Ulland BM, Weisburger JH, Yamamoto RS, et al: Antioxidants and carcinogenesis: Butylated hydroxytoluene, but not diphenyl-*p*-phenylenediamine, inhibits cancer induction by *N*-2-fluorenylacetamide and by *N*-hydroxy-*N*-2-fluorenylacetamide in rats. Food Cosmet Toxicol 11:199–207, 1973

(*10*) Grantham PH, Matsushima T, Mohan L, et al: Changes in the metabolism of labelled acetanilide and binding of isotope to serum and liver macromolecules during chronic administration. Xenobiotica 2:551–565, 1972

(*11*) Grantham PH, Mohan L, Yamamoto RS, et al: Alteration of the metabolism of the carcinogen *N*-2-fluorenylacetamide by acetanilide. Toxicol Appl Pharmacol 13: 118–130, 1968

(*12*) Grantham PH, Weisburger JH, Weisburger EK: Effects of the antioxidant butylated hydroxytoluene (BHT) on the metabolism of the carcinogens *N*-2-fluorenylacetamide and *N*-hydroxy-*N*-2-fluorenylacetamide. Food Cosmet Toxicol 11:209–217, 1973

(*13*) Weisburger JH, Shirasu Y, Grantham PH, et al: Chloramphenicol, protein synthesis, and the metabolism of the carcinogen *N*-2-fluorenyldiacetamide in rats. J Biol Chem 242:372–378, 1967

(*14*) Mohan LC, Grantham PH, Weisburger EK, et al: Mechanisms of the inhibitory action of *p*-hydroxyacetanilide on carcinogenesis by *N*-2-fluorenylacetamide or *N*-hydroxy-*N*-2-fluorenylacetamide. J Natl Cancer Inst 56:763–768, 1976

(*15*) Weisburger JH, Yamamoto RS, Williams GM, et al: On the sulfate ester of *N*-hydroxy-*N*-2-fluorenylacetamide as a key ultimate hepatocarcinogen in the rat. Cancer Res 32:491–500, 1972

Discussion II[1]

P. Magee: My remarks are concerned with work in the intact animal that I think is extremely important because everyone recognizes that to determine the molecular mechanisms of carcinogenesis, we first have to study the in vitro systems. Nevertheless, sooner or later, we always must come back to the intact animal to apply any concept that emerges from the in vitro work because it is the animal that gets the cancer and not the cell, bacterium, or chemical, as Kennaway remarked years ago.

Having said that, I thought that what I might try to do is suggest three topics of interest that might emerge in the general discussion and that you might like to raise with the speakers.

The first idea that I thought might be clarified by this sort of meeting is the fact that there are two types of carcinogens, the so-called genotoxic and the epigenetic.

This idea, I think, was first put forward by Druckery some years ago at a meeting in Sweden and has been taken up extensively more recently by several people, notably by Dr. Gary Williams, who is participating in this meeting.

By a genotoxic carcinogen, I am referring to the kind of carcinogen that has been so extensively studied by Drs. James and Elizabeth Miller, i.e., the type that produces an electrophile and reacts with cellular constituents, including the genetic material.

There is another type, which is becoming of greater interest that does not seem to do this. I am referring to compounds about which we have heard much, such as phenobarbitone, saccharin, several of the chlorinated hydrocarbons, and recently, the work of Alan Poland with tetrachlorodibenzodioxin. He has shown, I think rather convincingly, that it does not react with DNA, even the material with the highest specific activity he has.

Perhaps we might think a little about these two types of carcinogenic action and about how they may relate to the papers we have been hearing.

The second point is that the whole concept of specificity of targets for chemical carcinogens has to be studied, I think, in the intact animal because we could not get this information from any in vitro system.

Broadly speaking, the organ specificity of carcinogens depends 1) on the distribution of the "precarcinogen" (I am using the Millers' terminology, which everybody knows) whether it is concentrated in a particular organ or is uniformly distributed throughout the body, and 2) obviously on the metabolism of the precarcinogen, whether we have activating metabolism to form the proximate and ultimate carcinogens or deactivation metabolism to produce noncarcinogenic and more readily excreted metabolites. Then we have the crux of the matter, which is the reaction of the proximate and ultimate carcinogens with cellular constituents. Here we can have carcinogenic interactions, leading to the initiation of tumor formation (I am using the term "initiation" here now in the technical sense). We also have noncarcinogenic interactions, i.e., all the others that steer the electrophile away from where it can do the damage.

At this point, I think I would have to declare that most people now regard the initiating reaction as a reaction of DNA. If it is not, there is no point in implying the importance of DNA repair in carcinogenesis. I think everybody does this. We can perhaps consider that the interaction with DNA is essential for carcinogenesis, but you may wish to argue about that.

We have the possibility of repair of the initiating event, in this instance, the DNA adduct. Finally, as we have seen in several of the presentations, we have to have replication of the initiated cells to form the clones. There is a lot of evidence, as you know, that cancer results from clones of single initiated cells.

Finally, the third important concept is that of promotion, which again has been brought out extremely well in this meeting. Whatever may have been said until recently about initiation and promotion being shown only in the mouse skin under the correct circumstances, we now have heard about at least two systems: the liver system and the bladder system. The conditions are so close to mouse skin that we would have to say that initiation and promotion do occur in the liver and in bladder, and, therefore, perhaps by extrapolation, everywhere else.

Now perhaps Dr. John Weisburger might like to answer some questions. I was quite interested, Dr. Weisburger, when you were speaking of the importance of N-oxidation; you were also raising the question of epoxidation in carcinogenesis by these arylamines. You went on, a little later, to indicate that somehow the bacteria may play a role favoring an epoxidation mechanism. Could you explain that in greater detail?

J. Weisburger: First, as always, Dr. Magee, you have really summarized essentially what all of us have said. Certainly, the target specificity that you discussed has to do with the triplet or quadruplet, i.e., activation mechanisms, repair mechanisms, replication, all of which are essential. Promotion may be involved in fostering replication. This is how one would wish to address this question.

When Elizabeth Weisburger, Preston Grantham, and we studied the metabolism of acetanilide some years ago, we published a paper on this in *Xenobiotica*. We found that

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; OH = hydroxy; NCI = National Cancer Institute; DEN = diethylnitrosamine; DMBA = 7,12-dimethylbenz[*a*]anthracene; FANFT = N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; AHH = aryl hydrocarbon hydroxylase.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

one metabolite, i.e., 4-hydroxy-3-mercapturic acid could be construed to arise from a 3,4-epoxy. Certain of the data in the literature, e.g., the Millers' data presented here, that when 2-FAA is fed and the mouse skin is promoted with croton oil, 2-FAA seemed to be a better initiator than *N*-OH-2-FAA. It can also be construed to mean that maybe an epoxy derivative of 2-FAA may be the intermediary in some way.

The way to study it is for somebody to feed labile 2-FAA and then isolate the DNA adduct and see what it looks like, whether it is similar to the C-8 conventional product that has been isolated from liver or whether it is different. That is the way to proceed. Dr. Fiala in our laboratory is doing just that with some of the simpler arylamines.

The bacterial flora implications simply came from this, Dr. Magee, and that is based on the experiment we did many years ago in germfree animals when we found that when germfree rats were fed *N*-OH-2-FAA, one could actually find *N*-OH-2-FAA glucuronide excreted in the stools. However, in conventional animals, one found only 2-FAA in the stools, so that the normal conventional microbial flora contains glucuronidase splitting the glucuronide and an *N*-OH-dehydroxylase, if you will, to convert it back to 2-FAA. We are faced with the question: How do certain of these arylamines, the 3-methyl-4-aminobiphenyl compounds, react to result in colon cancer?

Again, we have some idea, on the basis of work of Dr. Fiala, that these metabolites are partly generated in the liver, excreted in the bile, and end up in the gut. We have no data as yet as to the nature of the metabolites in the gut, but if the 2-FAA example that I gave you earlier holds, one would think that any *N*-OH-glucuronide secreted would necessarily be converted back to the amine. One is then left with the question: How does the large bowel activate these chemicals. One can speculate that it could be by the epoxidation mechanism, rather than by N-oxidation, although that is also possible.

May I also add one model to the initiation–promotion concept you did not mention, i.e., that we have shown that bile acids were good promoters in colon carcinogenesis. Therefore, we have liver in the Peraino model, bladder in the Cohen model, and the bile acids promoting colon cancer, which we believe is extremely important in the human context.

Magee: Certainly, my apologies. Are there any more questions for Dr. Weisburger?

J. W. Gorrod: My comment is in regard to the methyl activating effect and those Williams' papers, which appeared about 1954. It struck me that there were other things apart from methyl groups that were activated. I think *o*-chlorine also showed some activity. Yet when you went to the iodo form, the fluoro activator was inhibited. I may be wrong. You must know this more than I do.

Other compounds, such as the Hackman compounds, have methoxy groups next to them. I think the methyl is not unique in being an activator. In fact, some people even think a hydroxy group next to the amine may activate. I think other groups are present as well.

J. Weisburger: Dr. Gorrod, you are one of the key experts in aromatic amine metabolism. I agree with you that (as I mentioned in the latest NCI bioassay report) methoxy *o*-anisidine is a much better urinary bladder and general carcinogen than is *o*-toluidine. Again, Dr. Fiala and his staff in our Institute are working on the mechanism whereby this happens, and we cannot get any answers yet. This is ongoing research.

You are right. It was Wolpole, not Williams, who did all of this synthetic work and tested these materials and found they caused colon cancer.

I find it most fascinating. The reason we are so keen on this now is because this particular structure leads to diseases that are important in man, i.e., cancers of the colon and breast. The Wolpole compounds all do it, and other structurally active ones such as the dimethyl forms no doubt would do it too.

As far as I know, and somebody correct me, please, 1-methyl-2-naphthylamine has never been tested. You might remember that the 3-methyl-2-naphthylamine was synthesized many years ago by someone in California; perhaps Dr. Elizabeth Miller will remember. It was tested by Dr. Sinoi and by us at the same time. The chemical was supplied by somebody in California who felt that the *o*-methylamine would not be carcinogenic, and thus we would find an *o*-naphthylamine for industrial use that was not carcinogenic. However, they were wrong, because 3-methyl-2-naphthylamine was more active.

The 1-methylamine has not been tested. Again, as part of the NCI Bioassay Program, we tested 2-methyl-1-anthramine. 1-Anthramine, as I implied, should not be carcinogenic, the 2-methyl seemed to be. This latter compound requires a lot more work, and I hope that in the next few years we will learn something about it.

All I implied in our presentation is that, although all of us think in terms of N-oxidation, let us also remember that phenyl rings can also activate in another way. Let us do further investigation to detect whether this is so. We do not have the answer now.

Magee: I want to raise a question with Drs. Cameron and Farber about the circulation to these hyperplastic liver nodules. It has been known for years that liver cancer has an almost entirely, if not entirely, arterial circulation. What I want to know is: When does this start in the nodule, if at all, and how does it influence the entry into the nodule of molecules put into the gastrointestinal tract? Can you comment on that?

R. Cameron: Certainly. In our laboratory, we have done 2 studies using the model I presented, the first of which is unpublished. This work was in collaboration with Dr. Aaron Rappaport of the Physiology Department. Hirosuta perfused animals with nodules and with hepatomas with latex material. The casts (I think he had about 10 animals) show the arterial circulation clearly, which, even as early as the nodule stage, was greatly enhanced. If you studied these latex models, you would see primarily the arterial circulation.

I think the 2d study, which was done by Dennis Solt in collaboration with an immunologist (Dr. J. B. Hay) at the University, was published in 1977 in *Cancer Research.* They used microspheres labeled with strontium and perfused them into animals that had nodules at different stages. These nodules were induced by 2 or 3 single injec-

tions of DEN at high doses. Using these microspheres, they found striking reductions in the portal blood flow. They would inject the spheres into the portal or arterial circulation of approximately 20% of the control animals, and they would isolate the nodules and compare them with the surrounding tissue.

Heterogeneity in the arterial flow in these tumors was considerable. In some instances, fourfold increases were observed in the tumors compared with the surrounding tissues, and in others a reduction was seen. However, the portal circulation was clear; it was almost like portacaval shunting of these spheres by the tumors.

Magee: Does anyone have another question for Dr. Cameron?

B. Ketterer: You mentioned, Dr. Cameron, that you have 2-FAA-resistant cells, and you said one reason why they might be resistant is because the 2-FAA does not penetrate them. I would like to know what evidence you have for this.

Cameron: This is preliminary evidence. It was done in Philadelphia by Sally Hartman, who was Dr. Farber's technician at the time. They fed [^{14}C]2-FAA, as I recall, to the animals. Then they isolated nodules and surrounding tissues. After 18 hours of exposure, they found an 80% reduction in the labeled carcinogen within the nodule, as opposed to the surrounding tissue. A portion of this study was published in 1976 in *Cancer Research.* In part of this same study, adducts to DNA and RNA of protein were also investigated. With both 2-FAA and labeled dimethylnitrosamine, there was approximately a 50% reduction in the adducts in all of these macromolecules.

We are planning to conduct a more extensive study of the inhibition and also to combine this with the in vitro study we are working on now.

Ketterer: I was wondering whether you felt that the membrane permeability had changed toward 2-FAA because that seems most unlikely. The experiment you described could have so many explanations.

Cameron: Oh, absolutely.

Ketterer: For example, circulation, or sites for binding inside the cell, etc., could account for the changes; I wanted to have that clarified.

J. Scribner: I am wondering what objective evidence you have that, at the beginning of this 2-FAA treatment, you have 2-FAA-resistant cells.

Cameron: This is a functional definition, in the sense that if you do a partial hepatectomy and you see all the cells respond with proliferation, you add 2-FAA to the diet and then note almost total inhibition of hepatocytes. The bile duct cells still proliferate. When DEN is the initial model, as soon as you do the partial hepatectomy after the 2-FAA treatment, you see only these selected cells growing out. The earliest cells you can see are maybe a clone of a small group of 10 cells growing in the sphere. You see this growth under electron microscopy. We developed large millimeter block sections in which you can see these minute growing areas; that is why we say 2-FAA-resistant because some cells are growing, whereas the rest of them are not.

J. Scribner: The matter that concerns me is whether the effect 2-FAA has is not one of failing to act on certain cells that you see after the partial hepatectomy, but rather that it has the same effect on all cells and that this is manifested only in those certain rare events induced by the precarcinogen. I am thinking in particular of an experiment published by Dr. Boutwell in 1964, in which he divided an initiating does of DMBA into four subinitiating doses. The tumor incidence which he saw with the initiating does was high enough that he should have been able to see one-fourth of that tumor incidence with each of the lowest of the divided doses, which suggests that certain things had to be pyramided to reach the complete effect.

What I am thinking is that possibly in your system you could be adding new events to those originally perpetrated by the carcinogen, and that it is these reactions which lead to these few rare cells that produce colonies after the partial hepatectomy.

Cameron: As you know, Dr. Scribner, in vivo work is extremely difficult to keep clean and to make nice schematic diagrams. What we put our confidence in is the fact that the whole series of controls do not show this differential inhibition and differential growth of the foci; in other words, you give DEN and you do a partial hepatectomy, or you give DEN and the 2-FAA.

As to whether you are adding other events with the 2-FAA, I think this is probable. In fact, we showed it ourselves in the text-figures in which 2-FAA was the selecting agent as well as the initiator. You give a single dose of 2-FAA around the time of the partial hepatectomy, a dose not much different from the one the animal is getting in his diet, e.g., 25 or 50%. Obviously, the 2-FAA is initiating some other type of event.

That is, of course, why we have been looking for the last several years for effective substitutes that may selectively stimulate the initiated cell. If we could do that, it would be an ideal clean system. As you know, it is difficult to get a clean system.

J. Weisburger: As I recall, Maini and Stich in 1961 published a paper in the *Journal of the National Cancer Institute* on inhibition of mitotic rate in animals fed various liver carcinogens. How does this old work relate to your development?

Cameron: 2-FAA is another in the list of chemicals that are potent inhibitors of DNA synthesis and of cell proliferation in the liver. Unfortunately, all of them also seem to be potent carcinogens.

J. Weisburger: However, that bears on your material.

Cameron: Oh, of course, it does; but it would be nice if they found another chemical that was not carcinogenic.

J. Weisburger: Dr. Gershbein, in Chicago, published some papers similar to that of Maini and Stich on increase in liver size; he published tables of data with compounds that do just that. You might wish to look at Gershbein's work.

Cameron: I will.

Magee: Thank you very much, Dr. Cameron. Dr. Peraino, if I understood you correctly, you said that you never see any of your hyperplastic nodules that regress. This seems to be different from what Dr. Cameron was saying. Can you explain this apparent difference, if it really is a difference?

C. Peraino: Well, our treatment does not induce any liver damage. The carcinogenetic treatment we give is innocuous, I would say, and the promoting agent, being nonhepatotoxic at the doses we give, does not introduce this ancillary problem of creating regenerative hyperplasia as a result of compensatory regeneration after damage.

All we see are the initiated cells developing into tumors. I think we have sidestepped an intermediary stage, what is looked upon as an intermediary stage (but may really be a side road) of the production of these hyperplastic nodules.

Magee: Thank you. Any more questions for Dr. Peraino?

M. J. Blyberg: In your study in which you showed phenobarbital as a promoting agent in mice, you gave phenobarbital to a strain of mice that had a moderately low rate of development of lesions, and when you gave the phenobarbital, you got a 100% rate. In interpretations of carcinogenesis experiments, at least by the Food and Drug Administration, such an effect might be considered carcinogenic. I think you cannot lightly dismiss the experiment as necessarily failing to show an initiating effect.

Peraino: I think you are getting into political rather than scientific definitions of carcinogens.

Blyberg: No, I do not think so at all. I think the Agency uses fine consultants.

Peraino: I would be happy to consult with you on this. I think you have to define phenobarbital as the promoter, and my definition of a promoter is one which facilitates the expression of preexisting neoplastic information without altering the character of that information. What we see in the mice already susceptible to spontaneous tumorigenesis is an increase in the types of tumors that would ordinarily appear without the administration of this agent. Promoting activity does not appear in mice and rats susceptible to spontaneous hepatic tumorigenesis.

In effect, I think that DDT has been misclassified as a carcinogen, when, in fact, it probably is a promoter. It also has been used in animals susceptible to spontaneous tumorigenesis, and it increases the incidence of tumors in those animals.

From the standpoint of assessment of health risks, I think it is wise to be conservative and to consider these chemicals as agents which might increase the incidence of hepatic tumors and therefore constitute a health hazard. From a scientific standpoint, I think it clouds the issue to label these things as carcinogens or initiators, when, in fact, they are promoters. Mechanistic interpretation may be implied, as well as possibly the assessment of levels of risk or threshold levels. These issues should be clarified.

J. Weisburger: I think this is an area that demonstrates the extraordinary importance of Dr. Peraino's work. The statements that Dr. Magee made on discrimination between genotoxic agents and agents that work by an epigenetic mechanism are also important.

E. Miller: I want to point out what all of us, I think, realize: Our definitions are in a state of flux. What seemed so obvious 10 or 20 years ago, when the Delaney Amendment was passed, is not the way we see things now.

My personal view is that the chemicals that can induce tumors have at least two activities, that of initiation and promotion. As we learn more, we may refine our definitions.

Some chemicals may be almost pure initiators, as, for instance, ethylcarbamate which appears to be for the skin. Some compounds may be almost pure promoters, as the phorbol esters appear to be for the skin or maybe phenobarbital for the liver. If you apply polycyclic hydrocarbons to the skin, you can get initiation and promotion of complete carcinogenesis, or you can titrate that dose down and have only an initiating activity.

I think we are approaching the stage fairly soon when the regulatory agencies and their consultants can no longer do research on a chemical and then state whether it is or is not a carcinogen. They are going to have to look into these finer details, which will make life much more complicated initially, but, in the long run, I think the assessment of risk will be much more realistic.

W. Troll: The two states of carcinogenesis, promotion and initiation, have been emphasized considerably here. First I want to say that all the bladder carcinogens mentioned are initiators in mouse skin. We have recently shown that 2- and 1-naphthylamine (2-FAA was reported earlier) are initiators in mice; males respond more promptly than females. We can feel more comfortable when we are perhaps talking about the same thing. The same materials will work in the skin and the bladder and partially in the liver.

My main point is that I think one of the tests of distinguishing between the two is that they can be inhibited by different agents. As shown a long time ago by Tannenbaum, promotion can be inhibited by starvation of the animals, perhaps a simple way of doing studies. Recently, in my laboratory, we have shown that certain proteases inhibit the promotion; even more recently, the *cis*-retinoic acids and retinoids have appeared as inhibitors of promotion.

All of these are testable devices. In other words, I would expect that if you are studying promotion of any kind, these inhibitors may work.

I was a little disappointed that all the reports on inhibition were only about the first stage, which I would call the first initiation, when the carcinogen has to find its receptor. I think that may be fine, but I think it is unlikely that in the human situation we will be able to afford such niceties. We must inhibit the event at later stages, and some inhibitors of promotion which I mentioned may be useful.

Magee: Thank you. Are there any more questions for Dr. Peraino specifically? If not, we can proceed to Dr. Cohen's paper. I found the remarks we are making about the various enzyme activities in different parts of the kidney most interesting. The differences between the cortex and the medulla, in particular your comments about the cyclooxygenases are, I must confess, new to me. I wonder if you might expand on those topics.

S. Cohen: Basically, until recently, the kidney has been considered as a single organ, rather than the 3 or 4 organs that it really is. I want to emphasize the mixed function oxidase and the cytochrome P_{450} systems. Levels are high in the renal cortex, lower in the medulla adjacent to the cortex, and they are essentially absent in the papilla or the inner cortex. The reverse is true with the cyclooxygenase; it

is essentially absent in the cortex, present in low levels in the outer medulla or the portion next to the cortex, and present in high levels in the papilla, the portion of the kidney through which it passes before going into the renal pelvis as urine.

Whether these have biologic meanings with regard to activation of carcinogens, I do not know. We are beginning to investigate that area.

That the activation with the cyclooxygenase system works for both benzidine and FANFT and that it is not just a process of metabolizing with regard to deactivation is interesting. If you use trapping, with the transfer RNA as the trapping nucleic acid, binding occurs to the nucleic acid as well.

H. Saxholm: Dr. Cohen, what fraction of the cells might be carrying these microvilli? Did you see any morphologic differences or were the microvilli uniform?

Cohen: I omitted the details on the pleomorphic microvilli because we are not absolutely certain our information is correct. At least during carcinogenesis induced by FANFT, the appearance of so-called pleomorphic microvilli correlates closely with the change from reversible to irreversible lesions. Once we see pleomorphic microvilli present on the cells, we always see them. They are present on all bladder tumor cells in the animal model and also in humans.

The uniform microvilli, which you may be talking about, are what are normally present on the underside of the superficial cells and on all sides of the intermediate and basal cells of the urinary bladder epithelium. These are short microvilli that are of uniform length, shape, size, and distribution. They cover a nice feltwork of the bladder epithelium. Occasionally, especially when you get glandular transformation, you can have so-called uniform long microvilli; again, these are uniform in length and shape, and they are straight microvilli.

The pleomorphic microvilli that we are talking about are truly pleomorphic because they vary in size and shape; many are bifurcated or trifurcated, and many have clubbed ends. They seem to be representative at least of some kind of abnormal proliferative process but not necessarily neoplasia.

Gorrod: You mentioned the role of urine. The ascorbic acid in urine seems to be involved (Linus Pauling will be pleased). Would you like to comment on the work of Schlagel and Pitkin?

You also talked about getting your active chemicals at the right place. Now, I do not know about this cyclooxidase. I know there are 5 or 6 N-oxidases that have various functions. One method used a long time ago by Jal, then Bunser, Clayson, and Boyland, was to insert the chemicals in the bladder. Had you thought of doing that kind of an experiment? I know it received some criticism. Nevertheless, it did generate a lot of data considered valuable at that time. I wonder if you would like to comment on either of those points.

Cohen: First of all, I do not know which of the cyclooxygenases is the important one, or if any of them are.

Gorrod: Can you define "activate"? You said that benzidine was activated.

Cohen: I said activated to a product that will bind to nucleic acid. That is what I mean by activation.

Although Dr. Jal is one of the major researchers who started putting chemicals into urinary bladders, the so-called pellet implantation technique, other articles have also appeared recently. One was in the 17th volume of an International Japanese Symposium, and the other is a recent paper in *Cancer Letters* by Dr. Clayson, which is essentially Jal's work which he published, that showed that the pellet alone (usually cholesterol, but occasionally paraffin) induced nearly 100% incidence of bladder tumors, if the pellet was left in and the animals were kept alive for at least 3 years. This is unlike the usual test during which the animals are killed at the end of 50 or 60 weeks. This model is potentially useful, but it is hard to interpret at this time.

Would I like to comment on the role of ascorbic acid? No, but I will anyway. If you look at the data of Schlagel and his colleagues, you find that, using the pellet implantation technique with anthranilic or hydroxyanthranilic acid as the chemical carcinogen and high levels of ascorbic acid, the development of these tumors in mice is inhibited.

However, if you look carefully at his data and apply more appropriate statistical tests than he does, the inhibition is not statistically significant. Also, the pellet implantation technique may not be appropriate. Others have now investigated ascorbic acid in both mice and rats in their attempts to inhibit urinary bladder carcinogenesis. One chemical used was FANFT (I worked on this with Mark Soloway when he was in Cleveland), and we found no inhibition. No one has been able to reproduce Schlagel's work.

Unidentified participant: One of your observations was that, if you gave more than one initiator in your system sequentially, the response was greater. In the real situation, people usually are exposed to more than one of these compounds. I think it is significant that you demonstrated the effect, and it is also significant that the regulatory agencies concerned with exposure to chemicals do not know how to address this to develop proper guidelines.

Cohen: I do not know how to address the question either, other than I would like to emphasize the fact that the data was Dr. Ito's and not mine.

S. Swaminathan: With reference to the nitrofuran metabolism, we do know a number of enzyme systems are involved, such as xanthine oxidase and NADPH cytochrome c reductase, etc. Do we have any specific information on the distribution of this enzyme in the medulla and inner medulla and more specific evidence of the cyclooxygenase? Is the effect we see a result of the activation of these other enzymes?

Cohen: Again, it seems like the activation of bladder carcinogens or any carcinogen involves some kind of oxidase or peroxidase system, and it does not seem to make much difference which one is used, whether it is the mixed function oxidase system, the cyclooxygenase system, peroxidase, or xanthine oxidase. A variety can be used. I think at this point that we do not know which one is important in any given situation. It may be that if one is inactivated, another takes over. The distribution of the cyclooxygen-

ase, as I said, is the highest in the inner medulla. Cyclooxygenase is also present in the urinary bladder, as is xanthine oxidase in the microsomal or P_{450} system.

Magee: I think this must be the last question.

Blyberg: In connection with the cyclooxygenase, a symposium was held at the Natick Laboratories (November 1979) on oxidaton processes with regard to fats and oils. Of course, oxidation products of unsaturated fats have been shown to be implicated with colon cancer. Scientists at Natick were interested in the possible oxidation products of unsaturated fatty acids. When unsaturated fatty acids were fed in the expectation that atherosclerotic deposits would be reduced, some evidence suggested that colon cancer developed. I do not know how true that is.

Oxidation products of cholesterol also attract attention. I think the implantation of pellets in the bladder might be considered as a possible synergistic mechanism, i.e., the cholesterol oxides and the test material might be present in the pellets.

Magee: Thank you. Does anyone want to reply to that?

Cohen: Dr. Weisburger, would you like to reply on the statement about colon cancer?

Magee: I think we should discuss Dr. Malejka-Giganti's paper. I was surprised to be informed that there is no P_{450} in mammary glands. Would this imply that in the classical Huggins' system for inducing mammary tumors, i.e., the hydrocarbon that he gave in the single dose, the hydrocarbon would be converted to an epoxide or some proximate carcinogen and then moved to the mammary gland?

D. Malejka-Giganti: It is hard for me to discuss in detail the DMBA-induced mammary tumorigenesis. DMBA, no matter what the route of administration is, systemic or topical, will induce mammary tumors.

Whether DMBA alone or its metabolite is active in mammary gland tumorigenesis has not been resolved. There are interesting data from tissue culture experiments, in which you have the transformation by DMBA of rat mammary epithelial cells or you have the transformation of a mammary organ culture by submerging the culture in the medium with DMBA, with development later of tumors in the isologous host, as shown by Dao's group.

Recently, Nandi's group found that, if you apply DMBA in the cultures, the rat mammary epithelium metabolizes DMBA to water-soluble metabolites. This was discovered by measurement of the radioactivity between the lipid and aqueous layers. The metabolites were not identified yet; whether they are active mammary gland carcinogens is not now known. There is still a possibility that the original Dao's hypothesis that the accumulation of DMBA alone in the fat pad will eventually initiate mammary gland tumorigenesis is true.

M. J. Griffin: Because I actually did this experiment, I think maybe it is time for me to speak up. We were interested in the mammary gland conversion of DMBA, i.e., of DMBA to water-soluble products. I knew the liver system and could do that well; it followed all the rules.

Dr. Margaret King, who has the finesse to get all these mammary glands isolated, did this work. I do not have that. She gave me microsomes and then I went to work.

I could not get any requirement for NADPH. I think the term should not be "cyclo," which is a specialty term, but "cooxygenase." In other words, you are not converting a hydride ion on NADPH to OH, like you do with P_{450}. You are just using the other oxygen, oxidized arachidonic acid, a polyunsaturated fat; that is a specialty term called cyclooxygenase.

It is cooxygenation here that I think is important in colon and mammary gland cancers. We should know what we are looking for. In other words, you get stimulation by some unsaturated compound and get the oxidation of the precarcinogen through at least a proximal carcinogen or even an ultimate carcinogen.

Boyland has some A-ring oxidations that I think go a long way toward indicating that Huggins' model is not inconsistent with what we have said about avoiding use of a P_{450} system and still getting oxidized parent carcinogens.

Nelson: I think several groups monitored AHH activity with mammary gland tissue. One group is Bresnick and co-workers in Vermont and another is in Montreal. In one of their recent articles, they mentioned having measured P_{450} activity in mammary gland microsomes. Actually, it is a different spectra that they have obtained.

I think it is still unclear whether P_{450} is there or not, and whether there is an NADPH requirement. Apparently, it is required for AHH activity.

Malejka-Giganti: May I comment? Do you refer to AAH activity in the mouse mammary gland?

Nelson: This is in the mouse.

Malejka-Giganti: Yes, AHH activity is more active in the mouse mammary gland than in that of a rat. With today's development, I think that we cannot identify AHH activity with N-hydroxylating activity. As a number of papers have now shown on the heterogeneous population of the cytochromes P_{450}, AHH activity is apparently associated with different cytochromes than N-hydroxylating activity. That would be for liver microsomes. If we assume that in the mammary gland we do not have cytochrome P_{450} identical to its hepatic form, hydroxylation of carcinogens could be mediated by other heme proteins or enzymes, as Dr. Griffin pointed out.

R. Floyd: Dr. Malejka-Giganti, have you used antibodies to see if indeed any P_{450} is there. I would like to add that it is my opinion that P_{450} is converted to P_{420} by detergent molecules; therefore, a lot of fat is in the mammary gland. I was wondering if this is what could be happening in your isolation procedure.

Malejka-Giganti: We do not use any detergent in this preparation. We used three methods for preparation of microsomes from mammary glands and exactly the same method for preparation of the liver microsomes; they were always parallel. We disrupted mammary tissue by collagenase, freezing, and pulverization in liquid nitrogen or homogenization of fresh tissue and spinning off the fat at low speed at 0° C. It is possible that the P_{420} comes from P_{450}, but we did not study that aspect.

Magee: We have time for only one more question.

Gorrod: The previous speaker was talking about aromatic arylamines, and Dr. Giganti is talking about aromatic arylamides. Maybe this microphone converts everything into arylamines, but there is a difference between an arylamine and an arylamide in chemistry, bio-

chemistry, toxicology, and pharmacology. I think sometimes these two expressions have been mixed up, which has caused a lot of confusion in the literature in the past. I would hope to think that at this meeting, at least, we would keep to one or the other, when appropriate.

Magee: Thank you, Dr. Gorrod. Do you want to reply to that, Dr. Giganti?

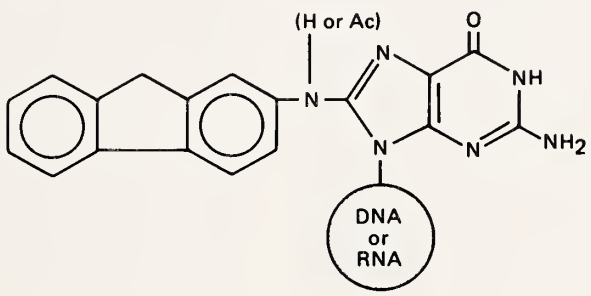
Malejka-Giganti: I thought we were talking about AHH activity and N-hydroxylating activity. We are not confusing amides with amines.

Gorrod: I was not raising the objection to Dr. Giganti, but generally.

Magee: Of course not. We must now discuss Dr. Grantham's paper. I think you said that [^{3}H]acetanilide became bound to DNA, and yet I think you also said this is not a carcinogen. Can you clarify these two observations?

P. Grantham: Yes, I did say that we showed that [^{3}H]acetanilide was bound to DNA. Of course, I have no evidence that it is not a carcinogen; some other colleagues might.

Session III: Animal Studies: Metabolism



Session Chairman: George T. Bryan

Discussion Chairman: James R. Gillette

Metabolic Studies In Vivo With Arylamines[1]

Elizabeth K. Weisburger[2]

ABSTRACT—Metabolic experiments have furnished leads on both the detoxification and activation pathways for aromatic amines. These consist largely of C- and N-hydroxylation, followed by conjugation with glucuronic or sulfuric acid, although other mechanisms may be involved. Current emphasis is on the N-hydroxylated derivatives as a precursor to the activated carcinogen. Metabolic patterns have furnished leads in some cases to account for the differences in susceptibility of various species to the carcinogenic aromatic amines, but discrepancies also occur.—Natl Cancer Inst Monogr 58: 95–99, 1981.

Studies of the in vivo metabolism of many compounds have mainly revealed the character of their detoxification products as excreted in the urine. However, identification of urinary metabolites has afforded insight into the activation pathways for carcinogenic aromatic amines.

Various research teams have devoted much effort to elucidating the metabolites of 2-NA (*1*) and 2-FAA (*2–4*); also, interest has been renewed in the metabolism of benzidine (*5*). However, the earliest attempt at identification of the urinary metabolites of a carcinogenic aromatic amine such as 2-NA was that of Wiley (*6*), who used an extraction procedure to isolate the sulfate of 1-OH-2-NA from the urine of dogs fed 2-NA. Bielschowsky (*7*) applied a similar technique to obtain a major metabolite, 7-OH-2-FAA, from the urine of rats fed 2-FAA (*7*). Other metabolites were neither identified nor recognized.

With the introduction of paper chromatography, the task of separation and eventual identification of the metabolites of foreign compounds became easier. Thus by application of several solvent systems (table 1), separation of at least 12 possible ring-hydroxylated metabolites of 2-FAA was accomplished (*8*). Additionally, the separation process was simplified by differential extraction of urinary metabolites into a water-immiscible solvent such as ether. Determinations of the extractable metabolites before and after the proper enzymic treatments for the liberation of glucuronides or sulfate conjugates afforded a measure of the relative levels of these conjugates. Material which was not extractable after these procedures was termed "water-soluble" (text-fig. 1). These latter metabolites may represent mercapturic acid derivatives and other unknown materials.

Similarly, Boyland and associates used differential precipitation and then paper chromatography to separate and identify metabolites of 2-NA in various species (*9–11*). As a corollary, the need for reference materials led to the synthesis of many previously unknown derivatives of 2-FAA or 2-NA, especially ring-monohydroxylated compounds, some of their esters, and mercapturic acids (*2, 4, 12*).

Furthermore, application of the paper chromatographic systems developed to separate metabolites of 2-FAA facilitated the separation and identification of the N-OH-derivative, a more active form than the ring-hydroxylated compounds (*3*). This development caused renewed interest in the metabolism of aromatic amines. A fair number of additional amines besides 2-FAA were investigated; they had the same N-hydroxylation pathway (*1, 3, 13*). However, the N-OH-metabolite has not yet been identified for some carcinogenic aromatic amines, i.e., 6-aminochrysene, 2,4-diaminoanisole, and 2,4-toluenediamine (*14–16*). Whether this lack is absolute or whether technical difficulties have prevented identification of the N-OH-derivatives remains to be determined.

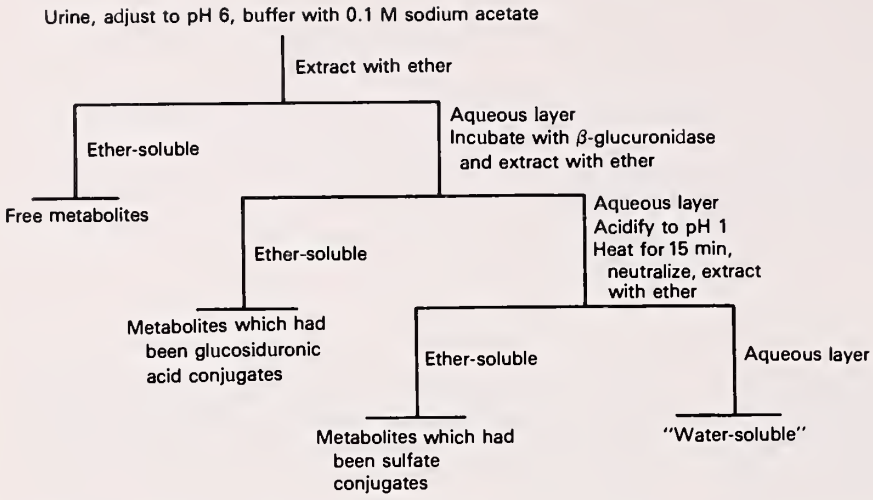
The solvent systems so useful for paper chromatography were also applied in column chromatography to separate larger quantities of the various metabolites. Ion-exchange chromatography, developed to separate urinary metabolites of differing polarity (*17*), indicated the possibility of 20 metabolites of 2-FAA in rats alone (text-fig. 2 and table 2). Likewise, Boyland and associates (*9–11*) demonstrated numerous urinary metabolites of 2-NA in the rat and rabbit (table 3). The multiplicity of urinary metabolites of 2 simple compounds indicated that various enzymatic and conjugating systems in the mammalian organism deal with these foreign compounds.

Further advances in analytical and separation technology have led to three types of chromatography: 1) thin-layer, 2) gas–liquid, and 3) high-pressure (performance) liquid. These techniques, judged from the number of reports in this area (*4*), have all been applied in the separation of metabolites of aromatic amines, but the third appears to be the most versatile and most useful of all. The application of immunologic techniques has also resulted in the development of a radioimmunoassay for 2-FAA–DNA adducts (*18*). Due to its sensitivity and specificity, this assay has facilitated research on the interaction of activated metabolites of 2-FAA with nucleic acids (*19*).

Abbreviations: 2-NA = 2-naphthylamine; 2-FAA = *N*-2-fluorenylacetamide; OH = hydroxy; 2-FA = 2-fluorenamine.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Laboratory of Carcinogen Metabolism, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Public Health Service, U.S. Department of Health and Human Services, Bethesda, Maryland 20205.

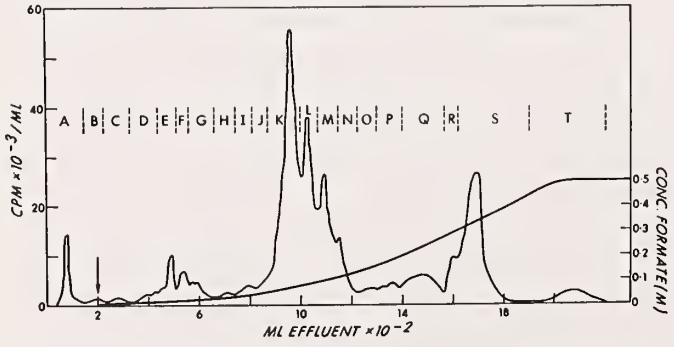


TEXT-FIGURE 1.—Fractionation scheme for classification of urinary metabolites of 2-FAA.

TABLE 1.—*Mobility of ring-hydroxylated metabolites of 2-FAA*

Compound	R_F value (×100) in solvent system			
	1[a]	2[b]	3[c]	4[d]
2-FAA	98–98	90–97	57–72	48–64
1-OH-2-FAA	78–86	92–96	50–64	50–64
3-OH-2-FAA	79–88	91–95	30–45	39–52
5-OH-2-FAA	88–93	87–92	12–20	13–25
6-OH-2-FAA			3–14	
7-OH-2-FAA	81–87	84–89	4–10	5–12
8-OH-2-FAA	84–90	87–91	6–14	8–17
2-FA	90–97	89–91	38–46	75–88
1-OH-2-FA	dec.	84–88	5–13	44–59
3-OH-2-FA	93–98	84–88	5–13	44–59
5-OH-2-FA	84–93	84–88	1–7	25–41
7-OH-2-FA	78–85	30–85	1–4	11–22
8-OH-2-FA	81–88	85–88	2–6	21–34

[a] Solvent 1 was *sec*-butanol, 3% ammonium hydroxide (3:1, vol/vol).
[b] Solvent 2 was *tert*-butanol, formic acid, water (70:15:15, vol/vol).
[c] Solvent 3 consisted of the top layer of cyclohexane, *tert*-butanol, glacial acetic acid, and water (16:4:2:1, vol/vol).
[d] Solvent 4 consisted of the top layer of cyclohexane, *tert*-butanol, pyridine, water (16:2:2:1, vol/vol).



TEXT-FIGURE 2.—Chromatographic separation of urinary metabolites of 9[^{14}C]-labeled 2-FAA on a DEAE-cellulose column. The sample (2.5 ml urine diluted 1:1 with H_2O) contained 10 × 10^6 counts/min and recovery of isotope was 92%. *Arrow* indicates the start of the gradient.

TABLE 2.—*DEAE-cellulose column separation of urinary metabolites of 2-FAA*

Fraction[a]	Percentage of [^{14}C]-labeled urine			
	Total	Free	Glucuronides	Sulfates
A	2.8	1.8	0.2	0.1
B	0.5	0.4		
C	0.7	0.7		
D	1.5	1.3		
E	2.4	0.6	0.8	0.1
F	1.8	0.5	1.1	
G	2.3	1.0	0.9	
H	1.3	0.5	0.3	
I	1.8	0.5	0.8	0.1
J	1.9	0.3	1.2	0.1
K	25.3	0.8	23.0	
L	13.0	2.2	10.4	
M	9.0	0.2	1.1	6.3
N	4.8	0.2	0.4	3.2
O	1.1		0.2	0.5
P	2.6		0.4	1.4
Q	6.1	0.3	2.4	2.1
R	4.0	0.2	0.2	3.2
S	13.6			12.4
T	3.3			1.7
Total	99.8	11.6	43.3	31.2

[a] Aliquots from each fraction were buffered at pH 6 and extracted five times with ether (free). The aqueous layer was incubated with β-glucuronidase and extracted five times with ether (glucuronides). The remaining aqueous layer was subjected to mild acid hydrolysis (0.2 N HCl) for 15 min, cooled, neutralized with solid $NaHCO_3$, and extracted five times with ether (sulfates).

APPLICATION OF METHODS

N-2-Fluorenylacetamide

Of all the aromatic amines, the metabolism of 2-FAA was probably studied in the greatest number of species, susceptible and nonsusceptible to its carcinogenicity. Examination of the data shows some interesting trends and correlations and also some discrepancies which have not been explained (tables 4 and 5).

TABLE 3.—*Urinary metabolites of 2-NA*

Metabolite	Occurrence in[a]				
	Dog	Guinea pig	Hamster	Rabbit	Rat
2-NA				+	+
2-Acetamidonaphthalene				+	+
N-OH-2-NA	+	−	−	−	−
2-Naphthylamine-*N*-glucosiduronic acid				+	+
2-Naphthylsulfamic acid				+	+
2-Acetamido-1-naphthylglucosiduronic acid		+	+		+
2-Amino-1-naphthylsulfuric acid	+		+ (trace)	+ (trace)	+
2-Amino-1-naphthylglucosiduronic acid	+	+	+	+	+
2-Amino-1-naphthylmercapturic acid	+				+
2-Amino-6-naphthol				−	+
2-Acetamido-6-naphthol		+		+	+
2-Amino-6-naphthylglucosiduronic acid	+ (trace)			−	+
2-Amino-6-naphthylsulfuric acid	+ (trace)	+		−	+
2-Acetamido-6-naphthylglucosiduronic acid		+	+	+	+
2-Acetamidonaphthalene-6-mercapturic acid		+ (trace)		+	+
2-Acetamido-6-naphthylsulfuric acid			+	+	+
2-Acetamido-5,6-dihydro-5,6-dihydroxynaphthalene				?	?
2-Acetamido-5,6-dihydroxynaphthalene sulfuric ester				?	?
2-Acetamido-5,6-dihydroxynaphthalene glucosiduronic acid				?	?
2-Amino-1-naphthylsulfuric acid *N*-glucosiduronic acid				+	+
Bis(2-amino-1-naphthyl) hydrogen phosphate	+				

[a] + = positively identified; − = not found; ? = questionable.

TABLE 4.—*Excretion pattern of 2-FAA in various species*

Species	Percent of dose in 2 days		Urinary fraction, % of urine[a]		
	Feces	Urine	Free	Glucuronides	Sulfates
Cat	28	22	5	13	60–80
Dog	10–50	19	ND	ND	ND
Guinea pig	3–13	69–84	1.8	84	2
Hamster	3.5	90	3	81	3–10
Human	6	89	3.8	38–59	3–9
Mastomys	22	32–52	2.4–5	39–56	18–28
Monkey	3	97	2–22	69–86	3–5
Mouse	10	66	9.9	53	24
X/Gf mouse	12	70	6	54	25
Rat	24	48–65	6	27	16
Steppe lemming	4–13	65	6.0	52	18

[a] ND = not done.

TABLE 5.—*Urinary metabolites of 2-FAA in various species*

Species	Major metabolites in urine, % of dose		
	N-OH-2-FAA	7-OH-2-FAA	5-OH-2-FAA
Cat	1.5	38	2.1
Dog	5.2	0.7	—
Guinea pig	—	90	trace
Hamster	5–6	28–35	4–5
Human	4–14	25–30	1.5
Mastomys	2.3	8.7	3–7
Monkey	0.6–2.7	7.2–18	—
Mouse	1.8–3.5	20	3.4
X/Gf mouse	1.5	25	2
Rabbit	13	28	0
Rainbow trout	—	3	0.2
Rat	0.3–15	12	7
Steppe lemming	0.8	22	0.9

Most researchers assume that cats do not form glucuronides. Although cats excreted the greater proportion of a dose of 2-FAA as sulfate conjugates (sulfate fraction) in the urine, evidence was indisputable that they also excreted glucosiduronic acid conjugates of 2-FAA metabolites. Therefore, the cat can form glucuronides but not to the extent that other species do, which is an indication that its sulfotransferase activity is far greater than is the level of glucuronyl transferase.

From tables 4 and 5, one can discern that a susceptible strain of mouse and a resistant strain (X/Gf) had similar excretion patterns. Furthermore, the relative amounts of *N*-OH-2-FAA excreted were of the same order. Thus the resistance of X/Gf mice cannot be explained readily on the basis of excretion and metabolic patterns.

Among primates, the monkey excreted less of a dose as *N*-OH-2-FAA than did humans. Furthermore, monkeys excreted 97% of a dose of 2-FAA in the urine within 2 days, whereas the urinary excretion in humans was lower. One might surmise from these results that the monkey is less likely to develop tumors from exposure to 2-FAA than would humans. Long-term studies with monkeys show their resistance to large total doses of 2-FAA given over a period of years (*20*).

Guinea pigs and steppe lemmings are two species resistant to the effects of 2-FAA. Guinea pigs apparently do not convert 2-FAA to *N*-OH-2-FAA, but steppe lemmings have a limited capability to do so. Therefore, other factors may be involved in the resistance of steppe lemmings to 2-FAA.

The formation of numerous ring-hydroxylated products during the metabolism of 2-FAA may result from any of several mechanisms. Fukui et al. (*21*) proposed a radical one. The large amounts of the 7- and 5-OH-2-FAA formed, in contrast to the low levels of 6- and 8-OH-2-FAA, could also be explained through arene oxide inter-

mediates. Selective reaction of such intermediates could account for the large amounts of 7-OH- and 5-OH-FAA and the minor levels of 6-OH and 8-OH-FAA depicted in text-figure 3 (*4*).

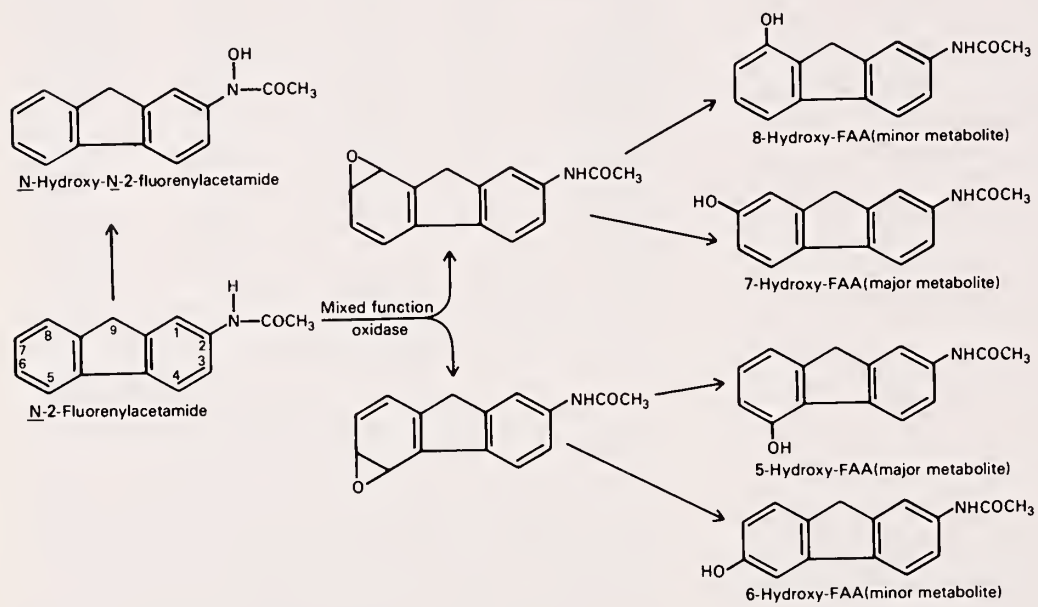
However, the *o*-amidophenols 1-OH-2-FAA and 3-OH-2-FAA seem to be formed by another mechanism, i.e., the isomerization of *N*-OH-2-FAA. Studies in vitro, as illustrated in text-figure 4, indicated that equal levels of each *o*-amidophenol are formed (*22*).

2-Naphthylamine

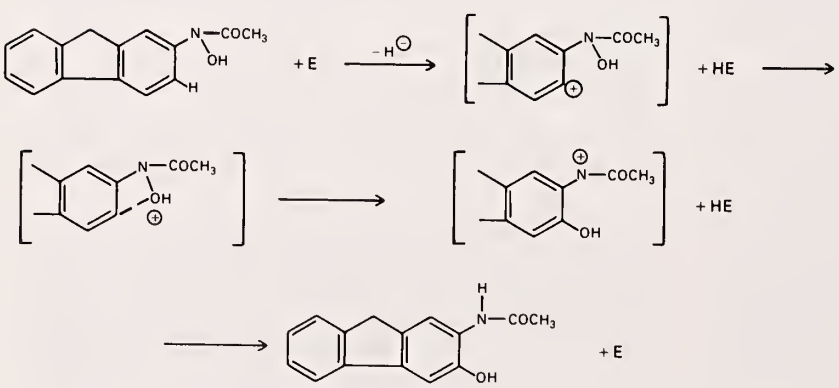
Boyland and Manson (*11*) published a comparative study of the urinary excretion of 2-NA metabolites by dogs, guinea pigs, hamsters, rabbits, and rats, all reportedly susceptible species. Paper chromatography was the primary means by which metabolites were separated. Of interest was the difference in patterns of metabolites within this group of animals (table 3). The dog was the only species to excrete *N*-OH-2-NA. Furthermore, the major metabolites were those hydroxylated in the 1-position, and only traces of 6-OH-derivatives were identified in the dog. With all other species, appreciable substitution occurred at both the 1- and 6-positions of 2-NA. The metabolites included free amines, acetylated amino and hydroxylated derivatives, sulfates or glucuronides of the hydroxy derivatives, and mercapturic acids. The greatest number of metabolites occurred in the rat, the species more likely to show liver than bladder tumors after having received 2-NA. Humans exposed to 2-NA also excreted *N*-OH-2-NA, which indicated a susceptibility and metabolic pattern like that of the dog (*23*).

Because the metabolites were mainly 1- or 6-derivatives of 2-NA with a small amount of a 5,6-dihydro-5,6-diol, one arene oxide intermediate was indicated (text-fig. 5), which may selectively afford 6-OH-2-NA. If the analogy with 2-FAA holds, the 1-OH-derivative might be formed through isomerization of an N-OH-derivative. If this were true, some *N*-OH-2-NA must be formed in all the species examined, inasmuch as all excrete some of the 1-hydroxylated metabolite. Therefore, the carcinogenicity of 2-NA in any one species may depend not only on the amount of *N*-OH-2-NA produced but also on the rate of isomerization to 1-OH-2-NA and subsequent conjugation.

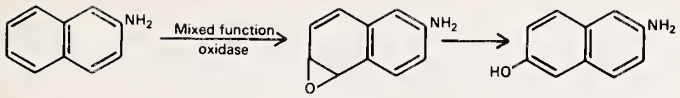
Unlike the situation with 2-FAA, 3-hydroxylated metabolites of 2-NA have not been reported. Whether this lack is due to a biologic deficit or a lack of interest is unknown.



TEXT-FIGURE 3.—Possible mode of formation of ring-hydroxylated derivatives of 2-FAA.



TEXT-FIGURE 4.—Possible mode of formation of *o*-amidofluorenols from *N*-OH-2-FAA.



TEXT-FIGURE 5.—Possible mode of formation of 6-substituted derivatives of 2-NA.

CONCLUSIONS

To summarize, knowledge of the metabolism of arylamines in vivo has afforded an insight into the presumed activation pathway of such compounds. However, excretion patterns do differ between susceptible species and between susceptible and nonsusceptible species; discrepancies also occur in the amounts of "activated" metabolite excreted by some susceptible and nonsusceptible species. Researchers need to find the explanations for these discrepancies, which eventually may afford means to inhibit or suppress the carcinogenicity of arylamines.

REFERENCES

(*1*) RADOMSKI JL: The primary aromatic amines: Their biological properties and structure-activity relationships. Annu Rev Pharmacol Toxicol 19:129–157, 1979

(*2*) WEISBURGER EK, WEISBURGER JH: Chemistry, carcinogenicity and metabolism of 2-fluorenamine and related compounds. Adv Cancer Res 5:331–431, 1958

(*3*) MILLER JA, MILLER EC: The metabolic activation of carcinogenic aromatic amines and amides. Prog Exp Tumor Res 11:273–301, 1969

(*4*) WEISBURGER EK: Laboratory chemicals: *N*-2-Fluorenylacetamide. *In* Carcinogens in Industry and the Environment (Sontag JM, ed). New York: Marcel Dekker, 1981, pp 583–666

(*5*) MORTON KC, KING CM, BAETCKE KP: Metabolism of benzidine to *N*-hydroxy-*N,N'*-diacetylbenzidine and subsequent nucleic acid binding and mutagenicity. Cancer Res 39:3107–3113, 1979

(*6*) WILEY FH: The metabolism of β-naphthylamine. J Biol Chem 124:627–630, 1938

(*7*) BIELSCHOWSKY F: A metabolite of 2-acetamidofluorene. Biochem J 39:287–289, 1945

(*8*) WEISBURGER JH, WEISBURGER EK, MORRIS HP: Urinary metabolites of the carcinogen *N*-2-fluorenylacetamide. J Natl Cancer Inst 17:345–361, 1956

(*9*) BOYLAND E, MANSON D: The biochemistry of aromatic amines. 4. *O*-Glucosiduronic acid derivatives of 2-naphthylamine. Biochem J 67:275–279, 1957

(*10*) ALLEN MJ, BOYLAND E, DUKES CE, et al: Cancer of the urinary bladder induced in mice with metabolites of aromatic amines and tryptophan. Br J Cancer 11:212–218, 1957

(*11*) BOYLAND E, MANSON D: The biochemistry of aromatic amines. The metabolism of 2-naphthylamine and 2-naphthylhydroxylamine derivatives. Biochem J 101:84–102, 1966

(*12*) BOYLAND E, MANSON D, NERY R: Biochemistry of aromatic amines. 9. Mercapturic acids as metabolites of aniline and 2-naphthylamine. Biochem J 86:263–271, 1963

(*13*) WEISBURGER JH, WEISBURGER EK: Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(*14*) GRANTHAM PH, GIAO NB, MOHAN LC, et al: The metabolism of 6-aminochrysene in the rat. Eur J Cancer 12:227–235, 1976

(*15*) GRANTHAM PH, BENJAMIN T, TAHAN LC, et al: Metabolism of the dyestuff intermediate 2,4-diaminoanisole in the rat. Xenobiotica 9:333–341, 1979

(*16*) GRANTHAM PH, MOHAN L, BENJAMIN T, et al: Comparison of the metabolism of 2,4-toluenediamine in rats and mice. J Environ Pathol Toxicol 3:149–166, 1980

(*17*) GRANTHAM PH: Separation of the urinary metabolites of *N*-2-fluorenylacetamide on columns of DEAE-cellulose anion exchanger. Biochem Pharmacol 16:429–440, 1967

(*18*) POIRIER MC, YUSPA SH, WEINSTEIN IB, et al: Detection of carcinogen-DNA adducts by radioimmunoassay. Nature 270:186–188, 1977

(*19*) POIRIER MC, DUBIN MA, YUSPA SH: Formation and removal of specific acetylaminofluorene-DNA adducts in mouse and human cells measured by radioimmunoassay. Cancer Res 39:1377–1381, 1979

(*20*) THORGEIRSSON SS, SAKAI S, ADAMSON RH: Induction of monooxygenases in rhesus monkeys by 3-methylcholanthrene: Metabolism and mutagenic activation of *N*-2-acetylaminofluorene and benzo[*a*]pyrene. J Natl Cancer Inst 60:365–369, 1978

(*21*) FUKUI K, NAGATA C, IMAMURA A, et al: Relationship between the electronic structure and carcinogenic activity of acetamidofluorene and related compounds. Gan 53:25–40, 1962

(*22*) GUTMANN HR, ERICKSON RR: The conversion of the carcinogen *N*-hydroxy-2-fluorenylacetamide to *o*-amidophenols by rat liver in vitro. Substrate specificity and mechanism of the reaction. J Biol Chem 247:660–666, 1972

(*23*) TROLL W, NELSON N: *N*-Hydroxy-2-naphthylamine, a urinary metabolite of 2-naphthylamine in man and dog. Fed Proc 20:41, 1961

Microsomal N- and C-Oxidations of Carcinogenic Aromatic Amines and Amides[1, 2]

Prabhakar D. Lotlikar *and* Young Sook Hong [3, 4]

ABSTRACT—Data on N-oxidation (activation step) and C-oxidation (inactivation step) of various carcinogenic aromatic amines and amides by liver microsomes from several species are reviewed. Cytochrome P_{450} involvement in both N- and ring-hydroxylation of *N*-2-fluorenylacetamide (2-FAA) is discussed. Data on reconstitution studies with rat and hamster liver microsomes indicated that all 3 components, cytochrome P_{450}, NADPH-cytochrome P_{450} reductase, and a lipid are required for N- and ring-hydroxylation of 2-FAA. Specificity in these oxidations is in the cytochrome P_{450} fraction.—Natl Cancer Inst Monogr 58: 101–107, 1981.

On the basis of epidemiologic data, aromatic amines such as 4-aminobiphenyl, 2-naphthylamine, and benzidine (text-fig. 1) have been responsible for the induction of bladder cancer among industrial workers (*1*). It is believed that these aromatic amines and their derivatives may account for a large proportion of all of the known incidence of industrial bladder cancer.

The requirement of large amounts of aromatic amines and amides for tumor induction and the appearance of tumors at sites distant from those of administration indicate that these compounds must be metabolized in any animal species before they are carcinogenic in that species (*2, 3*). Several excellent reviews on the metabolism of these carcinogenic compounds have appeared during the last decade (*2–11*). Three groups, Drs. James and Elizabeth Miller at the University of Wisconsin, Drs. John and Elizabeth Weisburger at the National Cancer Institute, and Dr. Helmut Gutmann at the University of Minnesota, have been pioneers in using 2-FAA as a model in the study of the mechanism of carcinogenesis by aromatic amines and amides. All species readily metabolize 2-FAA to yield non-carcinogenic monophenols which are excreted primarily as glucuronide and sulfate conjugates in the urine and bile (*2–8*). Since Cramer et al. (*12*) discovered the glucuronic acid conjugate of *N*-OH-2-FAA as a metabolite of 2-FAA in the rat, it has been demonstrated that all species susceptible to 2-FAA carcinogenesis excrete the N-OH-derivative of this carcinogen. Also, this derivative is more carcinogenic than 2-FAA in all susceptible species. Even in the guinea pig, which is resistant to carcinogenesis by this latter agent, *N*-OH-2-FAA is carcinogenic at the site of application [(*2, 3*) and references therein]. Thus unequivocally, N-hydroxylation is an activation step, whereas ring-hydroxylation is an inactivation step in carcinogenesis by 2-FAA and other aromatic amides and amines (*2–11*).

N- AND C-OXIDATION OF CARCINOGENIC AROMATIC AMINES AND AMIDES IN VITRO

Hydroxylation of 2-FAA at the 7-position was first demonstrated with rat liver microsomes in the presence of NADPH and O_2 (*13*) as shown in text-figure 2. Subsequently, hydroxylation reactions at other positions in the ring, i.e., 1, 3, and 5 were also shown with hamster (*14*) and rat (*15*) liver microsomes. In in vitro studies, pretreatment of rats with 3-MCA produced severalfold stimulation of ring-hydroxylations of this carcinogen (*15*). Recent studies (*16*) indicate that the C-9 position of 2-FAA is also hydroxylated by liver microsomes from various species. These investigators also observed that microsomal oxidation of the acetyl moiety of 2-FAA converted it to a glycolyl residue. Pretreatment of various species with either 3-MCA or PB shows different stimulatory responses to these oxidations (*16*).

Direct N-hydroxylation of 2-FAA was first demonstrated by Irving (*17*) with rabbit liver microsomes in the presence of NADPH and O_2. Since then, Irving (*18*) and others (*19–24*) have shown 2-FAA N-hydroxylation with liver microsomes from several species. Comparative metabolism with liver microsomes from various control and 3-MCA-pretreated species revealed marked differences in their ability to N- and ring-hydroxylate 2-FAA (table 1). In addition to a severalfold increase in various ring-hydroxylations as reported previously (*15*), pretreatment of rats with 3-MCA produced an increase in N-hydroxylation. On the other hand, 3-MCA induces in hamsters a specific and large increase in N-hydroxylation only. Patterns of N- and ring-hydroxylation in the control and 3-MCA-pretreated mice and rabbits follow those observed in rats and ham-

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; *N*-OH-2-FAA = *N*-hydroxy-2-FAA; 3-MCA = 3-methylcholanthrene; PB = phenobarbital; CO = carbon monoxide.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported by Public Health Service grants CA10604 and CA12227 from the National Cancer Institute.

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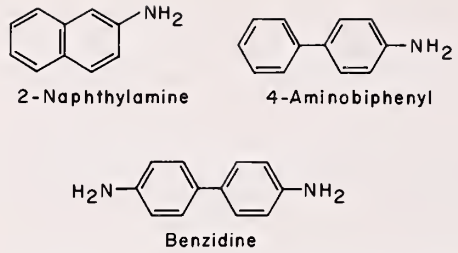
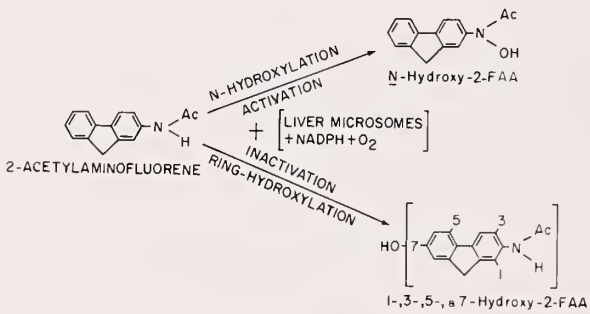


TEXT-FIGURE 1.—Human bladder carcinogens.



TEXT-FIGURE 2.—Metabolic hydroxylation of 2-FAA.

sters, respectively. In the guinea pig, even after such pretreatment, no N-hydroxylation could be detected, but recent studies by Gutmann and Bell (*22*) have shown trace amounts of N-hydroxylation activity with liver microsomes from this species. The specific increase in N-hydroxylation after pretreatment with 3-MCA of hamsters and rabbits appears to differentiate the N- and ring-hydroxylation systems. Furthermore, comparisons of the ratios of 3-, 5-, and 7-hydroxylation by liver microsomes from different species show that 7-hydroxylation activity varies independently of the 3- and 5-hydroxylations. These comparative studies supported the hypothesis that separate enzymes were involved in N- and C-hydroxylations of aromatic amines and amides.

Some of these in vitro induction effects afer 3-MCA pretreatment (table 1) have helped to explain in part the effects of its administration on the liver carcinogenicity of 2-FAA in rats and hamsters (*7*). However, a recent report (*25*) that pretreatment of rats with 2-FAA also promotes 2-FAA N-hydroxylation with liver microsomes suggests that this effect should be considered in the understanding of carcinogenesis induced by this compound.

In addition to 2-FAA, several other aromatic amines and amides are also N- and ring-hydroxylated by liver microsomes from various species (table 2). Some of the quantitative aspects of these studies need emphasis. Although Morton et al. (*31*) observed that the patterns of N- and ring-hydroxylations of *N,N'*-diacetylbenzidine with liver microsomes from control and 3-MCA-pretreated rats, mice, and hamsters resemble those 2-FAA studies presented in table 1, they did not detect the glycolamide derivative of the parent compound. However, glycolamides are formed with hamster liver microsomes with 4'-chloro- or 4'-fluoro-4-acetylaminostilbene but not with 4-acetylaminostilbene (*30*). The rate of N-hydroxylation of 4'-chloro and 4'-fluoro analogs with hamster liver microsomes was only 3% of that of the parent compound, 4-acetylaminostilbene (*30*). When rabbit liver microsomes were used, the 7-fluoro analog yielded two to three times more N-OH-metabolite as a substrate than 2-FAA (*17*).

TABLE 1.—*Effect of pretreatment with 3-MCA on the N- and ring-hydroxylation of 2-FAA by liver microsomes*[a]

Species	3-MCA treatment	nmol formed/g liver/20 min			
		N-OH-2-FAA	3-OH-2-FAA	5-OH-2-FAA	7-OH-2-FAA
Rat	–	ND[b]	24 ± 5	20 ± 4	57 ± 12
	+	37 ± 8	480 ± 100	395 ± 90	580 ± 190
Hamster	–	69 ± 18	47 ± 7	140 ± 19	640 ± 60
	+	950 ± 145	90 ± 21	186 ± 27	590 ± 130
Mouse	–	37 ± 6	31 ± 17	37 ± 5	120 ± 11
	+	236 ± 53	175 ± 33	220 ± 35	550 ± 210
Rabbit	–	16 ± 7	ND	ND	160 ± 85
	+	77 ± 15	"	"	200 ± 45
Guinea pig	–	ND	"	43 ± 15	1,150 ± 320
	+	"	"	73 ± 20	1,800 ± 300

[a] Data are from (*20*). Weanling male animals were given injections of 2–10 mg 3-MCA/100 g body wt 24 hr before assay for hydroxylase activity.

[b] ND = not detected.

TABLE 2.—*N- and ring-hydroxylation of carcinogenic aromatic amines and amides by liver microsomes*

Compound	Species	Oxidation N-	Oxidation C-	References
4-Aminobiphenyl	Rat	+		(*26*)
	Rabbit	+		(*19*)
	Dog	+		(*27*)
4-Acetylamino-biphenyl	Rabbit	+		(*19*)
	Dog	+		(*27*)
2-Aminofluorene	Rat		+	(*13*)
	Guinea pig and rat	+		(*28*)
7-Fluoro-FAA	Rabbit	+	+	(*17*)
	Hamster	+		(*20*)
2-Aminonaphthalene	Rat		+	(*13*)
	Rat	+		(*26*)
	Dog and rabbit	+		(*27*)
2-Acetylamino-naphthalene	Rat		+	(*13*)
	Dog and rabbit	+		(*27*)
4-Aminostilbene	Rat	+		(*26*)
4-Acetylaminostil-bene	Rat	+	+	(*29*)
	Hamster	+	+	(*30*)
N-Acetylbenzidine	Rabbit	+		(*19*)
N,N'-Diacetylben-zidine	Hamster, rat, and mouse	+	+	(*31*)
2-Acetylamino-phenanthrene	Hamster	+		(*20*)
N-Methyl-4-amino-azobenzene	Rat, guinea pig, hamster, mouse, and rabbit	+		(*32*)

ROLE OF CYTOCHROME P_{450} ENZYME SYSTEM IN 2-FAA N- AND RING-HYDROXYLATION

Differences in N- and ring-hydroxylation of 2-FAA and several other aromatic amides observed with liver microsomes from various species indicated that multiple enzymes are most probably involved in the oxidation of these compounds. At least 7 to 8 different cytochrome P_{450} are present in the mammalian liver microsomal cytochrome P_{450} enzyme system involved in the oxidation of physiologic and xenobiotic compounds (*33, 34*). Table 3 lists some of the evidence for involvement of the cytochrome P_{450} enzyme system in 2-FAA N- and ring-hydroxylation by liver microsomes. The first evidence was provided by Thorgeirsson et al. (*23*) and Gutmann and Bell (*22, 35*) who showed that 2-FAA N-hydroxylation by liver microsomes from various species was inhibited to a great extent in the presence of $CO-O_2$ mixtures. These investigators also demonstrated that this N-hydroxylation was inhibited in the presence of antibodies prepared against NADPH cytochrome c reductase (*23*). These observations have proved that the cytochrome P_{450} mixed function oxidase enzyme system is involved in 2-FAA N-hydroxylation. Our CO inhibition studies with hamster liver microsomes, prepared from controls and animals pretreated with 3-MCA demonstrating differences in inhibition of 2-FAA N- and ring-hydroxylation, suggested involvement of multiple forms of cytochrome P_{450} (*36*). Recently, Malejka-Giganti et al. (*25*) reported that $CO-O_2$ mixtures inhibited N-hydroxylation of 2-FAA by hepatic microsomes from rats treated with this compound.

Others (*22, 25, 37*) also demonstrated 2-FAA binding to cytochrome P_{450} that resulted in a characteristic type I binding spectrum. Differences in binding constants between species as well as between controls and animals pretreated with 3-MCA have been obtained (*22, 25, 37*). However, in view of the involvement of cytochrome P_{450} in both hydroxylation systems, it is not possible to correlate the magnitude of the substrate binding to the extent of either N- or ring-hydroxylation.

In contrast to N-hydroxylation of 2-FAA, that of *N*-methyl-4-aminoazobenzene is neither inhibited by $CO-O_2$ mixtures nor mediated by the cytochrome P_{450} enzyme system. N-Hydroxylation of this azo dye is mediated by a microsomal, mixed function amine oxidase which is a flavoprotein (*32*). According to Gorrod (*38*), the extent to which N-oxidation occurs in a species depends on the pK_a of the substrate; a lower pK_a favors oxidation by the cytochrome P_{450} enzyme system and a higher one by the amine oxidase system.

TABLE 3.—*Evidence for involvement of cytochrome P_{450} enzyme system in 2-FAA N- and ring-hydroxylation by liver microsomes*

Evidence	Species	Treatment	References
CO inhibition	Mouse and hamster		(*23*)
	Rat		(*22, 35*)
	Rat	3-MCA or 2-FAA	(*25*)
	Hamster	3-MCA	(*36*) [a]
NADPH cytochrome c reductase antibody inhibition	Hamster		(*23*)
Substrate binding	Rat and guinea pig		(*22*)
	Rat	3-MCA, PB, or 2-FAA	(*25*)
	Hamster	3-MCA	(*37*)

[a] N- and ring-hydroxylation were assayed. In other studies, only N-hydroxylation was assayed.

RESOLUTION AND RECONSTITUTION OF THE CYTOCHROME P_{450} ENZYME SYSTEM

Coon and co-workers, who were responsible for pioneering studies in solubilization and resolution of the cytochrome P_{450} enzyme system from rabbit liver microsomes, have indicated that the enzyme system has 3 components: cytochrome P_{450}, NADPH cytochrome P_{450} reductase, and phosphatidylcholine [(*33*) and references therein]. Reconstitution experiments with both rabbit and rat liver microsomal systems have shown that these 3 components are required for the oxidation of many physiologic and xenobiotic compounds (*33, 34*).

We, in this laboratory, have also solubilized and resolved the liver microsomal cytochrome P_{450} enzyme from rats and hamsters (*37, 39, 40*); we first treated liver microsomes with a bacterial protease before their solubilization with Triton X-100. A large amount of NADPH cytochrome c reductase (>97%) is removed from the microsomes after protease treatment. The final recovery of cytochrome P_{450} after Triton X-100 solubilization and ammonium sulfate precipitation is about 25–30% of the starting material. For reconstitution studies, NADPH cytochrome c reductase (*41*) and lipid fractions (*42*) are prepared by the published procedures.

N- AND RING-HYDROXYLATION OF 2-FAA WITH A RECONSTITUTED ENZYME SYSTEM

Reconstitution studies with hamsters (table 4) demonstrated that both P_{450} and reductase fractions are required for N- and ring-hydroxylation activity. However, the degree of N-hydroxylation is determined by the source of cytochrome P_{450} fraction. Thus N-hydroxylation activity of P_{450} fraction from 3-MCA-treated hamsters is severalfold greater than that of the same fraction from controls; these activities are slightly higher than those of their respective activities in whole microsomes. In contrast to N-hydroxylation, the amount of ring-hydroxylation in these reconstituted systems is lower than that of their respective whole microsomes, especially with control P_{450} fractions.

Kinetic data with liver fractions from hamsters pretreated with 3-MCA have indicated that a reductase fraction containing about 200 nmol NADPH cytochrome c reduc-

TABLE 4.—*Requirements of various microsomal fractions from livers of control (C) and pretreated hamsters (MCA) for 2-FAA N- and ring-hydroxylation*[a]

Fractions added	nmol OH-2-FAA formed/20 min	
	N	Ring
P_{450} (C) or P_{450} (MCA)	<0.8	1.2
Reductase (C) or reductase (MCA)	<0.8	0.8
P_{450} (C) + reductase (C)	4.8	7.3
P_{450} (C) + reductase (C) + lipid (C)	6.1	5.4
P_{450} (C) + reductase (MCA)	4.8	4.7
P_{450} (MCA) + reductase (MCA)	30.6	12.3
P_{450} (MCA) + reductase (C)	33.4	13.6
P_{450} (MCA) + reductase (MCA) + lipid (MCA)	36.9	15.2

[a] Data are from (*37, 40*). Where indicated, fractions containing 3.2 nmol cytochrome P_{450}, 610 nmol NADPH cytochrome c reductase/min, and 2 mg lipid were added. Control microsomes (3.2 nmol P_{450}) formed 4.3 and 38.4 nmol N- and ring-OH-2-FAA, respectively, whereas under similar conditions, microsomes from animals pretreated with 3-MCA formed 31.5 and 41.4 nmol, respectively.

tase activity/min/nmol of cytochrome P_{450} is required for optimum N- and ring-OH-2-FAA formations (*37*).

Like hamster liver (table 4), rat liver microsomal reconstitution studies also indicate that the degree of hydroxylating activity is determined by the source of the cytochrome P_{450} fraction even though both the P_{450} and reductase fractions are required for hydroxylating activity (table 5). In these reconstitution experiments, *N*-OH-2-FAA formation with P_{450} fractions from control and pretreated rats is much higher than their respective whole microsomes, which may be due to the fact that cytochrome P_{450} is a rate-limiting component in the reconstituted system but not in the whole microsomes.

Mouse liver reconstitution data (Lotlikar PD, Wang TF: Unpublished observations) are similar to those obtained with rat liver (table 5). In reconstitution studies with liver microsomal fractions from guinea pigs pretreated with 3-MCA, only ring-hydroxylation could be demonstrated (Lotlikar PD, Wang TF: Unpublished data).

Several investigators have reported that in addition to cytochrome P_{450} and NADPH cytochrome c reductase, phospholipid is required for the oxidation of various xenobiotics [(*33, 34*) and references therein]. However, in our reconstitution studies with hamsters (table 4), rats (table 5), mice, and guinea pigs, our failure to demonstrate an absolute lipid requirement may be due to incomplete removal of phospholipid or Triton X-100 from the cytochrome P_{450} fractions. Under such conditions, Triton X-100 may substitute for phospholipid for 2-FAA hydroxylation.

In our previous studies, the lipid requirement for 2-FAA N- and ring-hydroxylation was demonstrated with butanol-extracted liver microsomes (*43*). The procedure for the isolation of the cytochrome P_{450} fraction for reconstitution studies has now been modified. Instead of Triton X-100, protease-treated microsomes are solubilized with cholate (*37, 44*), and, under these conditions, about 80% of the phospholipid is removed from cytochrome P_{450} fractions. With such P_{450} preparations, an absolute phospholipid requirement can be demonstrated. In a typical experiment (table 6), formations of both N- and ring-hydroxylation are increased severalfold in the presence of a synthetic lipid such as dilauroyl phosphatidylcholine.

When various synthetic phosphatidylcholines and detergents are tested (table 7), only the dilauroyl, Triton X-100, and Triton N-101 are highly effective in restoring hydroxylation. Like dilauroyl phosphatidylcholine, Triton X-100 and Triton N-101 have preferential stimulatory effect on N- rather than on ring-hydroxylation.

Thus we have shown that, in addition to cytochrome P_{448} and reductase fractions, a lipid component is required for 2-FAA N- and ring-hydroxylation. Similar results are obtained with cytochrome P_{450} fractions isolated from liver microsomes from control and PB-treated hamsters. However, the amounts of hydroxylating activities obtained with these P_{450} fractions are much less than those obtained with the P_{448} from MCA-treated hamsters. Our preliminary kinetic results in reconstitution studies indicated that the presence of either dilauroyl phosphatidylcholine or Triton X-100 is responsible in stimulating NADPH-dependent

TABLE 5.—*Requirements of various microsomal fractions from livers of control and pretreated (MCA) rats for 2-FAA N- and ring-hydroxylation*[a]

Fractions added	nmol OH-FAA formed/20 min	
	N	Ring
P_{450} (C) or P_{450} (MCA)	0.6	0.6
Reductase (C) or reductase (MCA)	0.2	0.1
P_{450} (C) + reductase (C)	2.3	1.9
P_{450} (C) + reductase (MCA)	1.7	1.3
P_{450} (MCA) + reductase (MCA)	16	32
P_{450} (MCA) + reductase (C)	12	23

[a] Data are from (*39*). Where indicated, fractions containing 3.0 nmol cytochrome P_{450} and 510 nmol NADPH cytochrome c reductase/min were added. Control microsomes containing 3.0 nmol cytochrome P_{450} formed 1.4 and 10 nmol of N-and ring-OH-2-FAA, respectively. Under similar conditions, liver microsomes from animals pretreated with 3-MCA formed 6.0 and 57 nmol, respectively.

TABLE 6.—*Phospholipid requirement for 2-FAA N- and ring-hydroxylation by cytochrome P_{450} fraction from liver microsomes of MCA-pretreated hamsters*[a]

Fractions added	nmol OH-2-FAA formed/30 min	
	N	Ring
P_{450} (0.25 nmol)	<0.1	<0.1
Reductase (100 nmol)	<0.1	<0.1
P_{450} + reductase	0.5	0.3
P_{450} + reductase + lipid[b]	3.1	1.7

[a] This is the modified procedure of Lotlikar et al. (*40*). Protease-treated microsomes are solubilized with 2 mg cholate/mg protein instead of Triton X-100. Microsomes containing 0.25 nmol cytochrome P_{450} formed 2.4 and 2.9 nmol of N- and ring-OH-2-FAA, respectively.

[b] Dilauroyl phosphatidylcholine (0.05 mg) was used.

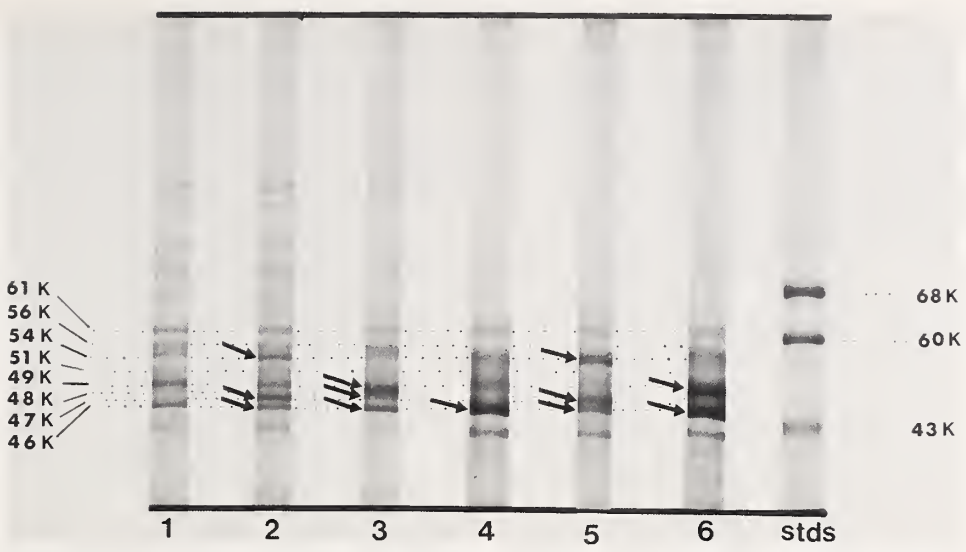


FIGURE 1.—Electrophoretogram of liver microsomal proteins from hamsters. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was done according to Laemmli (*47*), with a 7.5% separating gel and a 3% stacking gel in a discontinuous buffer system. Electrophoretic migration is from top to bottom. Total microsomal proteins containing 30 μg in 10 μl were applied in positions 1 to 3; microsomal proteins of cholate-solubilized cytochrome P_{450} fractions containing 60 μg in 20 μl were applied in positions 4 to 6. Standard proteins included ovalbumin, catalase, and bovine serum albumin with molecular weights of ~43,000, 60,000, and 68,000, respectively. Positions 1 and 4, 2 and 5, and 3 and 6 represent liver microsomal protein from control animals and hamsters pretreated with 3-MCA and PB, respectively. *Arrows* indicate Coomasie blue-stained protein bands of 46,000, 47,000, and 54,000 daltons in positions 2 and 5; 46,000 daltons in position 4; 46,000, 48,000, and 49,000 daltons in position 3; and 46,000 and 49,000 daltons in position 6.

reduction of cytochrome P_{448} (Lotlikar PD, Hong YS: Unpublished data).

Genetic studies with inbred mice have indicated that 3-MCA-inducible 2-FAA N-hydroxylase activity is associated with *Ah* locus (*45*). Induction of this enzyme activity in rabbits after treatment with 3-MCA closely parallels increases in an electrophoretic band of protein with a molecular weight of 54,000 (*46*). In our studies, sodium dodecyl sulfate gel electrophoresis of liver microsomes and cytochrome P_{450} fractions from control, 3-MCA-pretreated, and PB-pretreated hamsters showed great variations in protein bands (fig. 1). Thus in hamsters pretreated with 3-MCA, protein bands of 47,000 and 54,000 daltons are increased, and, based on catalytic activity for 2-FAA N-hydroxylation, it appears that protein bands of either weight or both may be responsible for the 2-FAA N-hydroxylation in these animals. Further purification of these and other cytochrome P_{450} would help the elucidation of the precise roles of these hemoproteins in N- and C-oxidations of various aromatic amines and amides.

TABLE 7.—*Effect of phospholipids and detergents on 2-FAA N- and ring-hydroxylation by reconstituted liver microsomes from MCA-pretreated hamsters*[a]

Phospholipid or detergent added	2-FAA hydroxylation, % of control	
	N	Ring
None	14	9
Dilauroyl phosphatidylcholine	82	33
Dimyristoyl "	16	10
Dipalmitoyl "	20	9
Distearoyl "	15	6
Dioleyl "	11	2
Triton X-100	87	35
Triton N-101	120	33
Cholate	25	9
Deoxycholate	30	12

[a] Data are from (*44*). Control microsomes containing 0.25 nmol cytochrome P_{448} formed 3.9 and 5.8 nmol *N*- and ring-OH-2-FAA, respectively. Cholate-solubilized 0.25 nmol P_{448} and 100 nmol NADPH cytochrome c reductase were used for reconstitution studies. Where indicated, 0.5 mg phospholipid or 0.1 mg detergent was added.

REFERENCES

(*1*) SCOTT TS: Carcinogenic and Chronic Toxic Hazards of Aromatic Amines. Amsterdam: Elsevier, 1962

(*2*) MILLER JA, MILLER EC: Activation of carcinogenic aromatic amines and amides by N-hydroxylation in vivo. *In* Carcinogenesis: A Broad Critique. Baltimore: Williams & Wilkins, 1967, pp 397–420

(*3*) _____: The metabolic activation of carcinogenic aromatic amines and amides. Prog Exp Tumor Res 11:273–301, 1969

(*4*) IRVING CC: Species and tissue variations in the metabolic activation of aromatic amines. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 211–227

(*5*) _____: Conjugates of N-hydroxy compounds. *In* Metabolic Conjugation and Metabolic Hydrolysis (Fishman

WH, ed), vol 1. New York: Academic Press, 1970, pp 53–119

(6) Miller JA, Miller EC: Ultimate chemical carcinogens as reactive mutagenic electrophiles. *In* Origins of Human Cancer (Hiatt HH, Watson JD, Winsten JA, eds), vol B. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1977, pp 605–627

(7) Clayson DB, Garner RC: Carcinogenic aromatic amines and related compounds. *In* Chemical Carcinogens, American Chemical Society Monogr No. 173 (Searle CE, ed). Washington, D.C.: Am Chem Soc, 1976, pp 366–461

(8) Weisburger JD, Weisburger EK: Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(9) Kiese M: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines and mechanisms of ferrihemoglobin formation. Pharmacol Rev 18: 1091–1161, 1966

(10) Arcos JC, Argus MF: Chemical Induction of Cancer. Structural Bases and Biological Mechanisms, vol IIB. New York: Academic Press, 1974

(11) Kriek E: Carcinogenesis by aromatic amines. Biochim Biophys Acta 355:177–203, 1974

(12) Cramer JW, Miller JA, Miller EC: N-Hydroxylation: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J Biol Chem 235: 885–888, 1960

(13) Booth J, Boyland E: The biochemistry of aromatic amines. 3. Enzymic hydroxylation by rat liver microsomes. Biochem J 66:73–78, 1957

(14) Seal US, Gutmann HR: The metabolism of the carcinogen *N*-(2-fluorenyl) acetamide by liver cell fractions. J Biol Chem 234:648–654, 1959

(15) Cramer JW, Miller JA, Miller EC: The hydroxylation of the carcinogen 2-acetylaminofluorene by rat liver: Stimulation by pretreatment in vivo with 3-methylcholanthrene. J Biol Chem 235:250–256, 1960

(16) Benkert K, Fries W, Kiese M, et al: *N*-(9-Hydroxy-9H-fluoren-2yl)acetamide and *N*-(9-oxo-9H-fluoren-2yl) acetamide: Metabolites of *N*-(9H-fluoren-2yl)acetamide. Biochem Pharmacol 24:1375–1380, 1975

(17) Irving CC: N-Hydroxylation of the carcinogen 2-acetylaminofluorene by rabbit liver microsomes. Biochim Biophys Acta 65:564–566, 1962

(18) _____: Enzymatic N-hydroxylation of the carcinogen 2-acetylaminofluorene and the metabolism of *N*-hydroxy-2-acetylaminofluorene-9-^{14}C in vitro. J Biol Chem 239:1589–1596, 1964

(19) Booth J, Boyland E: The biochemistry of aromatic amines. 10. Enzymic N-hydroxylation of arylamines and conversion of arylhydroxylamines into *o*-aminophenols. Biochem J 91:362–369, 1964

(20) Lotlikar PD, Enomoto M, Miller JA, et al: Species variations in the *N*- and ring-hydroxylation of 2-acetylaminofluorene and effects of 3-methylcholanthrene pretreatment. Proc Soc Exp Biol Med 125:341–346, 1967

(21) Enomoto M, Sato K: N-Hydroxylation of the carcinogen 2-acetylaminofluorene by human liver tissue in vitro. Life Sci 6:881–887, 1967

(22) Gutmann HR, Bell P: N-Hydroxylation of arylamides by the rat and guinea pig. Evidence for substrate specificity and participation of cytochrome $P_{1\text{-}450}$. Biochim Biophys Acta 498:229–243, 1977

(23) Thorgeirsson SS, Jollow DJ, Sasame HA, et al: The role of cytochrome P_{450} in N-hydroxylation of 2-acetylaminofluorene. Mol Pharmacol 9:398–404, 1973

(24) Matsushima T, Grantham PH, Weisburger EK, et al: Phenobarbital-mediated increase in ring- and N-hydroxylation of the carcinogen *N*-2-fluorenylacetamide and decrease in amounts bound to liver deoxyribonucleic acid. Biochem Pharmacol 21:2043–2051, 1972

(25) Malejka-Giganti D, McIver RC, Glasebrook AL, et al: Induction of microsomal N-hydroxylation of *N*-2-fluorenylacetamide in rat liver. Biochem Pharmacol 27: 61–69, 1978

(26) Uehleke H: N-Hydroxylation of carcinogenic amines in vivo and in vitro with liver microsomes. Biochem Pharmacol 12:219–221, 1963

(27) Brill E, Radomski JL: Comparison of the in vitro and in vivo N-oxidation of the carcinogenic aromatic amines. Xenobiotica 1:347–348, 1971

(28) Kiese M, Renner G, Wiedemann I: N-Hydroxylation of 2-aminofluorene in the guinea pig and by guinea pig liver microsomes in vitro. Arch Exp Pathol 252:418–423, 1966

(29) Baldwin RW, Smith WR: N-Hydroxylation in aminostilbene carcinogenesis. Br J Cancer 19:433–443, 1965

(30) Gammans RE, Sehon RD, Anders MW, et al: Microsomal N-hydroxylation of trans-4′-halo-4-acetamidostilbenes. Drug Metab Dispos 5:310–316, 1977

(31) Morton KD, King CM, Baetcke KP: Metabolism of benzidine to *N*-hydroxy-*N,N′*-diacetylbenzidine and subsequent nucleic acid binding and mutagenicity. Cancer Res 39:3107–3113, 1979

(32) Kadlubar FF, Miller JA, Miller EC: Microsomal N-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Cancer Res 36:1196–1206, 1976

(33) Coon MJ, Vermilion JL, Vatsis, et al: Biochemical studies on drug metabolism. Isolation of multiple forms of liver microsomal cytochrome P_{450}. *In* Drug Metabolism Concepts (Jerina DM, ed), Am Chem Soc Symp. Ser. No. 44. Washington, D.C.: Am Chem Soc, 1977, pp 46–71

(34) Lu AY, Levin W: The resolution and reconstitution of the liver microsomal hydroxylation system. Biochim Biophys Acta 344:205–240, 1974

(35) Gutmann HR, Bell P: Specificity of N-hydroxylation of arylamides. Fed Proc 32:665, 1973

(36) Lotlikar PD, Zaleski K: Inhibitory effect of carbon monoxide on the *N*- and ring-hydroxylation of 2-acetamidofluorene by hamster hepatic microsomal preparations. Biochem J 144:427–430, 1974

(37) Lotlikar PD, Hong YS, Baldy WJ Jr: Cytochrome P_{450}-dependent *N*-hydroxylation of 2-acetylaminofluorene. *In* Biological Oxidation of Nitrogen (Gorrod JW, ed). Amsterdam: Elsevier, 1978, pp 185–193

(38) Gorrod JW: Differentiation of various types of biological oxidation of nitrogen in organic compounds. Chem Biol Interact 7:289–303, 1973

(39) Lotlikar PD, Zaleski K: Ring- and *N*-hydroxylation of 2-acetamidofluorene by rat liver reconstituted cytochrome P_{450} enzyme system. Biochem J 150:561–564, 1975

(40) Lotlikar PD, Luha L, Zaleski K: Reconstituted hamster liver microsomal enzyme system for *N*-hydroxylation of the carcinogen 2-acetylaminofluorene. Biochem Biophys Res Commun 59:1349–1355, 1974

(41) Lu AY, West S: Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds and endogenous substrates. III. Properties of the reconstituted 3,4-benzpyrene hydroxylase system. Mol Pharmacol 8:490–500, 1972

(*42*) Roerig DL, Mascaro L Jr, Aust SD: Microsomal electron transport: Tetrazolium reduction by rat liver microsomal NADPH-cytochrome c reductase. Arch Biochim Biophys 153:475–479, 1972

(*43*) Lotlikar PD, Dwyer EN, Baldy WJ, Jr, et al: Phospholipid requirement for 2-acetamidofluorene *N*- and ring-hydroxylation by hamster liver microsomal cytochrome P_{450} enzyme system. Biochem J 168:571–574, 1977

(*44*) Hong YS, Lotlikar PD: Lipid requirement for 2-acetylaminofluorene (AAF) *N*- and ring-hydroxylation by reconstituted hamster liver microsomal enzyme system. Proc Am Assoc Cancer Res–Am Soc Clin Oncol 19:128, 1978

(*45*) Thorgeirsson SS, Nebert DW: The *Ah* locus and the metabolism of chemical carcinogens and other foreign compounds. Adv Cancer Res 25:149–193, 1977

(*46*) Atlas SA, Boobis AR, Felton JS, et al: Ontogenetic expression of polycyclic aromatic compound inducible monooxygenase activities and forms of cytochrome P_{450} in rabbit. Evidence for temporal control and organ specificity of two genetic regulatory systems. J Biol Chem 252:4712–4721, 1977

(*47*) Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685, 1970

Glucuronide Formation in the Metabolism of N-Substituted Aryl Compounds[1, 2]

Charles C. Irving[3]

ABSTRACT—*N*-Glucuronide conjugates of *N*-arylhydroxylamines and *O*-glucuronides of *N*-acetyl-*N*-arylhydroxylamines may play significant roles in the extrahepatic carcinogenicity of arylamines by serving as stable transport forms of metabolically activated precursors. In extrahepatic target organs, these glucuronides can undergo pH-dependent hydrolytic reactions to liberate an activated metabolite or one capable of being further activated.—Natl Cancer Inst Monogr 58: 109–111, 1981.

Glucuronidation reactions are important in the metabolism of N-substituted aryl compounds. Only the glucuronides that involve the nitrogen function of aromatic amines will be considered here (text-fig. 1): *O*-glucuronides of *N*-acetyl-*N*-arylhydroxylamines (I); *O*-glucuronides of *N*-arylhydroxylamines (II); *N*-glucuronides of *N*-arylhydroxylamines (III); and *N*-glucuronides of arylamines (IV).

O-GLUCURONIDES OF *N*-ACETYL-*N*-ARYLHYDROXYLAMINES

Glucuronides of *N*-acetyl-*N*-arylhydroxylamines are quantitatively the most important type involved in the excretion of N-hydroxy metabolites of aromatic amines or amides in most species. *N*-Acetyl-*N*-arylhydroxylamines are in the urine and bile after administration of the corresponding aglycone or the amine or amide precursor, almost entirely as conjugates yielding the corresponding aglycone when the urine or bile is treated with β-glucuronidase (*1, 2*). Several of these glucuronides have been isolated or synthesized (*1–3*).

The principal route of biosynthesis of these glucuronides involves UDPGA and a microsomal glucuronyl transferase. For example, we demonstrated formation of the glucuronides of *N*-OH-2-FAA, *N*-OH-AABP, *N*-OH-AAS, and *N*-OH-AAP from the corresponding N-hydroxy compound by rat or mouse liver microsomes in vitro in the presence of UDPGA (*2, 3*). Species variation in hepatic glucuronyl transferase activity with *N*-OH-2-FAA as a substrate is shown in table 1.

Although *O*-glucuronides of *N*-acetyl-*N*-arylhydroxylamines are stable metabolic products at neutral pH, they are labile at slightly alkaline pH due to the migration of the N-acetyl group to the 2′-hydroxyl of the glucuronyl moiety (*1, 4*). This reaction leads to the formation of the unstable *O*-glucuronide of the corresponding *N*-arylhydroxylamine. This type of glucuronide also displayed unusual and unexpected reactivity with a number of nucleophiles (*1, 3, 7*). The *O*-glucuronide of *N*-OH-2-FAA (*N*-OGl-FAA) was the most reactive compound of this type, but it had a much lower rate of reaction with nucleic acids or proteins than did *N*-AcO-2-FAA or the *O*-sulfonate of *N*-OH-2-FAA. However, the reactivity of these glucuronides is enhanced markedly at a higher pH, and the enhanced reactivity appears to be due to the generation of the *O*-glucuronide of the *N*-arylhydroxylamine under slightly alkaline conditions. The reaction products are characterized by the loss of the N-acetyl group of the reacting glucuronide. The aryl group has a strong effect on the rate of reaction of this type of glucuronide with nucleophiles (*3*). A comparison of the rates of reaction of *N*-OGl-2-FAA with the glucuronides of *N*-OH-4-AAS (N-OGl-AAS), *N*-OH-4-AABP (*N*-OGl-AABP), and *N*-OH-2-AAP (*N*-OGl-AAP) with tRNA, rRNA, DNA, polyadenylate, polyguanylate, polyuridylate, and polycytidylate showed that the relative order of reactivity of these glucuronides with nucleic acids was *N*-OGl-2-FAA > *N*-OGl-AAS >*N*-OGl-AABP > *N*-OGl-AAP (this last compound had only marginal or negligible reactivity). Whereas reactivity of *N*-OGl-2-FAA was greater with polyguanylate than with polyadenylate, the reverse was true for *N*-OGl-AAS, and both had much less reactivity with polyuridylate and polycytidylate. Except for the low reactivity of *N*-OGl-AABP with polyadenylate, this glucuronide and *N*-OGl-AAP were nonreactive with the homopolynucleotides. The relative rates of the reactions of this series of glucuronides with nucleic acids parallel the hepatocarcinogenicity of their N-hydroxy compounds.

As much as 30% of a single oral dose of 2-FAA was excreted in 24 hours in the urine of rabbits as the glucuronide of *N*-OH-2-FAA (*8*). Interestingly, the epithelial lining of the urinary tract of the rabbit is one tissue of this

Abbreviations: UDPGA = uridine-5′-diphosphoglucuronic acid; *N*-OH-2-FAA = *N*-hydroxy-2-fluorenylacetamide; *N*-OH-AABP = *N*-hydroxy-4-acetylaminobiphenyl; *N*-OH-AAS = *N*-hydroxy-4-acetylaminostilbene; *N*-OH-AAP = *N*-hydroxy-4-acetylaminophenanthrene; *N*-AcO-2-FAA = *N*-acetoxy-2-FAA; *N*-OH-2-FA = *N*-2-fluorenylhydroxylamine.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported by the Veterans Administration and Public Health Service grant CA26165 from the National Cancer Institute.

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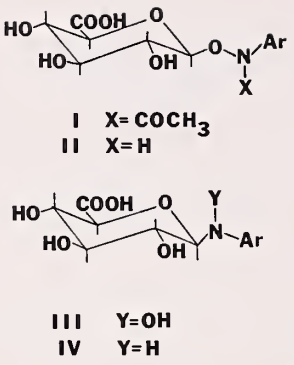


TEXT-FIGURE 1.—Types of glucuronide conjugates involving the nitrogen function of aromatic amines; Ar = aryl ring.

TABLE 1.—*N-OH-2-FAA glucuronyltransferase activity in liver microsomes from several rodent species*

Species	Sex	*N*-OH-2-FAA conjugated[a] (nmol/10 min/mg protein)
Hamster[b]	Male	39.4 ± 2.0
Mouse	"	36.2 ± 3.3
Rabbit	"	25.3 ± 1.7
Rat	Female	23.1 ± 0.6
Rat	Male	22.3 ± 1.2
Guinea pig[b]	"	12.9 ± 0.7

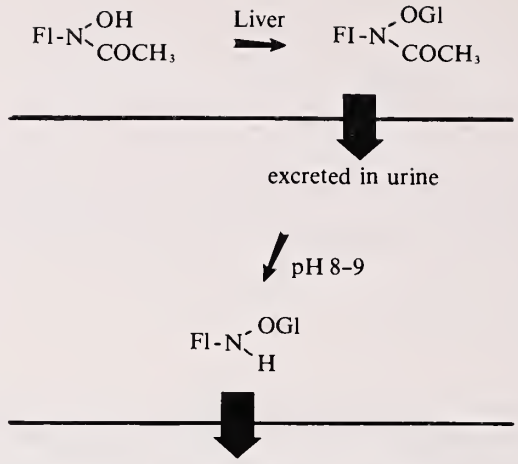
[a] The incubation mixture contained in a final volume of 0.33 ml: 20 μmol Tris-HCl, pH 7.4; 25 μmol sucrose; 0.42 μmol *N*-OH[9-^{14}C]FAA; 2.5 μmol UDPGA; and 0.25 mg microsomal protein. After incubation for 10 min at 37° C, 2 ml ice-cold 0.4 M sodium acetate, pH 6, was added. Unconjugated substrate was removed by extraction with ether, and bacterial β-glucuronidase was added. After 2 hr at 37° C, the *N*-OH-[9-^{14}C]FAA released was extracted into ether and counted. The identity of the glucuronide formed and the product that was released by hydrolysis with β-glucuronidase were independently confirmed (*3, 4*).

[b] Sodium fluoride at a final concentration of 0.1 M was added to inhibit the rapid deacetylation of *N*-OH-2-FAA by hamster and guinea pig liver microsomes (*5, 6*). This concentration of sodium fluoride had no effect on the glucuronyltransferase activity of rat liver microsomes.

species known to be susceptible to carcinogenesis by 2-FAA or *N*-OH-2-FAA (*9*). Inasmuch as the rabbit excretes a fairly alkaline urine (average pH is 8.5, but it frequently is as high as 8.9–9.0), one can speculate that the increased reactivity of the glucuronide of *N*-OH-2-FAA at pH 8.5–9.0 plays an important role in the susceptibility of the epithelium of the urinary tract of the rabbit to 2-FAA. A mechanism by which this might occur is shown in text-figure 2.

The glucuronide of *N*-OH-2-FAA, when administered sc, also induces a low incidence of tumors at the injection site and in the mammary gland, liver, and ear duct sebaceous gland (*10*).

Little information is available on the reactivity of *O*-glucuronides of other *N*-acetyl-*N*-arylhydroxylamines. Recently, Mulder et al. (*11*) reported that the *O*-glucuronide of *N*-hydroxyphenacetin formed in vitro led to covalent binding to protein in the system, and they suggested that this binding might play a role in the toxicity



TEXT-FIGURE 2.—Metabolic pathway postulated to be involved in the induction of bladder cancer in the rabbit by *N*-OH-2-FAA; Fl = 2-fluorenyl ring; Gl = glucuronyl moiety.

of high doses of phenacetin, especially in the kidney and bladder.

O-GLUCURONIDES OF *N*-ARYLHYDROXYLAMINES

To date we have no evidence that this type of glucuronide is formed enzymatically by conjugation of *N*-arylhydroxylamines. *O*-Glucuronides of *N*-arylhydroxylamines can be formed as intermediates resulting from chemical (*12*) or enzymatic (*13*) deacetylation of *O*-glucuronides of *N*-acetyl-*N*-arylhydroxylamines.

Two *O*-glucuronides of *N*-arylhydroxylamines have been reported in the literature. The *O*-glucuronide of *N*-OH-2-FA (*N*-OGl-2-FA) was prepared by the deacetylation of the *O*-glucuronide of *N*-OH-2-FAA (*12*). Synthesis of the *O*-glucuronide of *N*-4-biphenylylhydroxylamine by a similar method was reported by Radomski et al. (*14*). The *N*-OGl-2-FA is unstable in aqueous solution and reacts at neutral pH with nucleic acids, methionine, and tryptophan (*12*). *N*-OG1-2-FA reacts with guanosine-5′-monophosphate to give 8-(*N*-2-fluorenylamino)-guanosine 5′-monophosphate but does not react with the 5′-monophosphates of uridine, cytidine, or adenosine (*12*). When assayed with the use of the *Bacillus subtilis* transformation system, the mutagenic activity of *N*-OGl-2-FA equaled or surpassed that reported for *N*-AcO-2-FAA and the *O*-sulfonate of *N*-OH-2-FAA (*15*). We proposed that *N*-OGl-2-FA might be formed as a metabolite of *N*-OH-2-FAA by deacetylation of the *O*-glucuronide of *N*-OH-2-FAA and might account for the observed binding of *N*-OH-2-FAA to rat liver DNA in vivo (*1, 2*). Cardona and King (*13*) showed that the *O*-glucuronide of *N*-OH-2-FAA could be activated by deacetylation in guinea pig liver to give *N*-OGl-2-FA; the deacetylation of the glucuronide of *N*-OH-2-FAA could

be involved in the activation of this compound in extrahepatic tissues, including the bladder.

N-GLUCURONIDES OF *N*-ARYLHYDROXYLAMINES

Kadlubar et al. (*16*) reported that several *N*-arylhydroxylamines can be converted to their *N*-glucuronides by microsomes prepared from livers of dogs, rats, and humans. These *N*-glucuronides were stable and nonreactive at neutral pH. However, at pH 5, the *N*-glucuronides of *N*-1-and *N*-2-naphthylhydroxylamine and *N*-4-biphenylylhydroxylamine were rapidly hydrolyzed to the corresponding free arylhydroxylamines that were then converted to reactive derivatives capable of covalent binding to nucleic acids. This mechanism of reaction is distinct from that involving the *O*-glucuronide of *N*-OH-2-FA, which is highly reactive at neutral pH and for which reactivity does not proceed through the intermediate formation of the free *N*-OH-2-FA (*12*).

Although some evidence that this type of *N*-glucuronide is formed in vivo in the dog has been demonstrated (*14*), this has not yet been demonstrated in other species. The *N*-glucuronide of *N*-4-biphenylyhydroxylamine was isolated from the urine of dogs given 4-aminobiphenyl, and the conjugate was reported to be mutagenic at pH 5.5 or 6.8 in strains TA1538 and TA98 of *Salmonella typhimurium*. It has been proposed that *N*-glucuronides of this type serve as stable transport forms for carcinogenic aromatic amines. Kadlubar et al. (*16*) suggested that hydrolysis of these *N*-glucuronides and conversion of the resulting free arylhydroxylamines to reactive electrophilic arylnitrenium ions occur in the normally acidic urine of dogs and humans and that these reactions may be critical for the induction of bladder cancer with aromatic amines in these species.

N-GLUCURONIDES OF ARYLAMINES

N-Glucuronides of arylamines can be formed enzymatically, but the glucuronyltransferase is not identical with the enzyme(s) involved in the formation of *O*-glucuronides (*17, 18*). These acid-labile *N*-glucuronides can also be formed nonenzymatically by reaction of glucuronic acid with the free arylamine (*17, 18*). No good evidence is available to suggest that this type of glucuronide is of major importance in the metabolism of aromatic amines.

REFERENCES

(*1*) Irving CC: Conjugates of N-hydroxy compounds. *In* Metabolic Conjugation and Metabolic Hydrolysis, vol I (Fishman WH, ed). New York: Academic Press, 1970, pp 53–119

(*2*) _____: Metabolic activation of N-hydroxy compounds by conjugation. Xenobiotica 1:387–398, 1971

(*3*) _____: Influence of the aryl group on the reaction of glucuronides of *N*-arylacethydroxamic acids with polynucleotides. Cancer Res 37:524–528, 1977

(*4*) Hill JT, Irving CC: Biosynthesis and alkaline sensitivity of the NO-glucuronide of the carcinogen *N*-2-fluorenylacethydroxamic acid. Biochemistry 6:3816–3821, 1967

(*5*) Irving CC: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26:1390–1396, 1966

(*6*) _____: Species and tissue variations in the metabolic activation of aromatic amines. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 211–227

(*7*) Miller EC, Lotlikar PD, Miller JA, et al: Reactions in vitro of some tissue nucleophiles with the glucuronide of the carcinogen *N*-hydroxy-2-acetylaminofluorene. Mol Pharmacol 4:147–154, 1968

(*8*) Irving CC: *N*-Hydroxylation of 2-acetylaminofluorene in the rabbit. Cancer Res 22:867–873, 1962

(*9*) Irving CC, Wiseman R Jr, Young JM: Carcinogenicity of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene in the rabbit. Cancer Res 27:838–848, 1967

(*10*) Irving CC, Wiseman R Jr: Studies on the carcinogenicity of the glucuronides of *N*-hydroxy-2-acetylaminofluorene and *N*-2-fluorenylhydroxylamine in the rat. Cancer Res 31:1645–1648, 1971

(*11*) Mulder GJ, Hinson JA, Gillette JR: Generation of reactive metabolites of *N*-hydroxy-phenacetin by glucuronidation and sulfation. Biochem Pharmacol 26:189–196, 1977

(*12*) Irving CC, Russell LT: Synthesis of the *O*-glucuronide of *N*-2-fluorenylhydroxylamine. Reaction with nucleic acids and with guanosine monophosphate. Biochemistry 9:2471–2476, 1970

(*13*) Cardona RA, King CM: Activation of the *O*-glucuronide of the carcinogen *N*-hydroxy-*N*-2-fluorenylacetamide by enzymatic deacetylation in vitro: Formation of fluorenylamine-tRNA adducts. Biochem Pharmacol 25:1051–1056, 1976

(*14*) Radomski JL, Hearn WL, Radomski T, et al: Isolation of the glucuronic acid conjugate of *N*-hydroxy-4-aminobiphenyl from dog urine and its mutagenic activity. Cancer Res 37:1757–1762, 1977

(*15*) Maher VM, Reuter MA: Mutations and loss of transforming activity caused by the *O*-glucuronide conjugate of the carcinogen *N*-hydroxy-2-aminofluorene. Mutat Res 21:63–71, 1973

(*16*) Kadlubar FF, Miller JA, Miller EC: Hepatic microsomal *N*-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res 37:805–814, 1977

(*17*) Dutton GJ: The biosynthesis of glucuronides. *In* Glucuronic Acid, Free and Combined (Dutton GJ, ed). New York: Academic Press, 1966, pp 185–299

(*18*) Meittinen TA, Leskinen E: Glucuronic acid pathway. *In* Metabolic Conjugation and Metabolic Hydrolysis, vol I (Fishman WH, ed). New York: Academic Press, 1970, pp 157–237

Sulfation in the Metabolism and Mutagenicity of *N*-Hydroxy-2-fluorenylacetamide[1]

Jack A. Hinson,[2, 3] Larry S. Andrews,[4] *and* James R. Gillette[2, 5]

ABSTRACT—Sulfation has been previously implicated as an important step in the hepatocarcinogenesis of *N*-hydroxy-2-fluorenylacetamide (*N*-OH-2-FAA). Inasmuch as most mutagens are carcinogenic and most carcinogens are mutagenic, the role of sulfation in the mutagenesis of *N*-OH-2-FAA was investigated by the *Salmonella* mutagenesis system (TA1538) and a 100,000 × *g* rat liver supernatant previously chromatographed on Sephadex G-25 as a source of a sulfotransferase enzyme. In the absence of the supernatant, *N*-OH-2-FAA was only weakly mutagenic, whereas in the presence of a supernatant, a potent mutagen was formed. This mutagenicity was partly mediated by *N*-hydroxy-2-fluorenylamide (*N*-OH-2-FA) formed by esterases and acyl transferase. In the presence of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the sulfation cofactor, the carcinogen was only weakly mutagenic, and covalent binding to protein and DNA occurred. Mutagenicity increased approximately tenfold when *N*-OH-2-FAA, PAPS, and ascorbic acid were present. Ascorbic acid did not inhibit sulfation of *N*-OH-2-FAA, and no evidence could be found for the formation of *N*-OH-2-FA. An examination of the metabolites from *N*-2-FAA-sulfate indicated covalent binding to protein, and DNA as well as the formation of several other metabolites were blocked by ascorbic acid. However, the formation of 2-FAA, a nonmutagen, was increased approximately sixfold. Apparently, the only effect that ascorbic acid had was to reduce the reactive nitrenium ion metabolite to 2-FAA. Inasmuch as ascorbic acid would presumably reduce this reactive metabolite by sequential one-electron additions, it seemed possible that the mutagenic intermediate was a free radical of 2-FAA. Because the free radical metabolite is derived from the same reactive metabolite that was covalently bound to DNA, its formation in vivo apparently correlated with covalent binding to DNA, RNA, and protein in tissues.—Natl Cancer Inst Monogr 58: 113–116, 1981.

The carcinogenic activity of 2-FAA, which produces tumors in the liver, mammary gland, ear duct, and small intestine of the rat (*1–3*), is believed to be mediated primarily through the metabolite *N*-OH-2-FAA formed by a liver microsomal, cytochrome P_{450} mixed function oxidase (*4–6*). Even though *N*-OH-2-FAA is apparently an important step in the conversion of 2-FAA, there is evidence that it is further metabolized to produce several ultimate carcinogenic forms (*7–11*).

The staff of this laboratory has been interested in the role of sulfation in the metabolism and carcinogenicity of *N*-OH-2-FAA. Previous work by DeBaun and co-workers (*7*) suggested that the *N*-sulfate ester of *N*-OH-2-FAA (*N*-2-FAA-sulfate) may be an important metabolite in the hepatocarcinogenesis of *N*-OH-2-FAA and covalent binding of the 2-FAA moiety to cellular macromolecules. In their work, the hepatocarcinogenesis of *N*-OH-2-FAA in a number of different species correlated directly with in vitro sulfotransferase activity when *N*-OH-2-FAA was used as a substrate and with in vivo protein covalent binding of *N*-OH-2-FAA. These data suggested that sulfation of this hydroxy derivative led to the formation of a reactive metabolite, the aryl nitrenium ion, which could covalently bind to critical macromolecules; it was thus suggested that this metabolite was important in the hepatocarcinogenesis of 2-FAA (*1, 3*).

To characterize further the role of sulfation of *N*-OH-2-FAA in the metabolism and carcinogenicity of 2-FAA, we examined the mutagenicity of *N*-OH-2-FAA in the Ames *Salmonella* test system. This assay has yielded an excellent correlation between carcinogenicity and mutagenicity. Thus far, approximately 90% of the known carcinogens are mutagenic and 90% of the mutagens are carcinogenic (*12–14*).

Initially, a method was developed to generate enzymatically *N*-2-FAA-sulfate. A 100,000 × *g* supernatant fraction of rat liver, previously chromatographed on a Sephadex G-25 column to remove small molecular weight molecules, is used as the source of sulfotransferase enzyme. When catalytic amounts of PAP and *p*-nitrophenyl sulfate are added, the sulfate from the latter compound is enzymatically transferred to PAP and produces PAPS, the active sulfation cofactor, and *p*-nitrophenol. In the presence of *N*-OH-2-FAA, the sulfate of PAPS is enzymatically transferred to form *N*-2-FAA-sulfate and

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; *N*-OH-2-FAA = *N*-hydroxy-2-FAA; PAP = adenosine 3′,5′-diphosphate; PAPS = 3′-phosphoadenosine-5′-phosphosulfate: *N*-OH-2-FA = *N*-hydroxy-2-fluorenylamide.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

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PAP is regenerated. The rate of this reaction may be followed spectrophotometrically at 405 nm by the formation of *p*-nitrophenol. When [^{14}C]*N*-OH-2-FAA is used as a substrate protein, covalent binding from a metabolite produced from *N*-2-FAA-sulfate also may be used as an indirect assay (*15, 16*).

The mutagenicity of enzymatically generated *N*-2-FAA-sulfate was determined in Ames' bacterial tester strain TA1538. *N*-OH-2-FAA in the absence of the sulfation enzyme was only weakly mutagenic as Durston and Ames (*17*) reported (text-fig. 1). However, in the presence of a supernatant fraction without sulfation cofactors, a potent mutagen was formed. This mutagenicity is believed to be at least partly mediated by *N*-OH-2-FA, which is formed enzymatically by esterases (amidases) and acyl transferase (*18, 20*). Surprisingly, the addition of a sulfation cofactor decreased the mutagenicity (*21, 22*). This finding was unexpected because it appeared to be inconsistent with the view that metabolic activation of *N*-OH-2-FAA by sulfation is important in hepatocarcinogenicity.

In an effort to determine what role the reactive metabolite played in mutagenesis, we studied whether various compounds, such as DNA, RNA, guanine, and methionine that interact with the reactive metabolite, could decrease the remaining mutagenesis (*22*). Surprisingly, these compounds (tables 1 and 2) did not alter mutagenesis; but ascorbic acid or NADPH did increase it approximately tenfold. In the absence of supernatant enzymes, ascorbic acid was nonmutagenic, and it inhibited covalent binding of the reactive metabolite to protein as shown in table 2 (*16, 22–24*) and also binding to DNA (*25*).

To ensure that this increased mutagenesis was not the result of an inhibition of the sulfotransferase enzyme and thereby an increase in the formation of *N*-OH-2-FA by deacetylases and acyltransferase, we investigated the deacetylation of *N*-OH-2-FAA (table 2). Because the formation of [^{14}C]acetate from [^{14}C acetyl]*N*-OH-2-FAA represents the maximum amount of deacetylation, [^{14}C]-acetate formation was examined under the conditions described for the mutagenesis. In the presence of supernatant, [^{14}C]acetate was a metabolite of [^{14}C acetyl]*N*-OH-2-FAA (table 2). With a PAPS generating system, however, acetate was not a detectable metabolite either in the presence or absence of ascorbic acid (table 2). Also, ascorbic acid did not significantly alter the rate of disappearance of *N*-OH-2-FAA from the incubation mixture. Thus the increase in mutagenesis observed by the sulfation of *N*-OH-2-FAA in the presence of ascorbic acid apparently could not be explained by the formation of *N*-OH-2-FA (*21*).

We examined the total metabolism of [^{14}C acetyl]*N*-OH-2-FAA following sulfation in the presence and absence of ascorbic acid in our attempt to elucidate the metabolite that was responsible for the increased mutagenesis. Among the metabolites formed were: 2-FAA, a radiolabeled product covalently bound to protein, a dimer of 2-FAA, 1-(*N*-2′-fluorenylacetamido)-2-acetylaminofluorene (*24*), a phosphate conjugate of 7-OH-2-FAA, and a metabolite with a molecular weight corresponding to a *p*-nitrophenol adduct of 2-FAA. When ascorbic acid was included in the incuba-

TABLE 1.—*Effect on various experimental conditions on mutagenesis of* N-*OH-2-FAA*[a]

Additions	Buffer[b]	100,000 × *g* supernatant	100,000 × *g* supernatant plus PAPS
None	65	1,750	252
DNA	130	2,040	225
RNA	95	1,610	275
Guanine	91	1,740	196
Methionine	92		280
Ascorbic acid	92	2,896	2,910

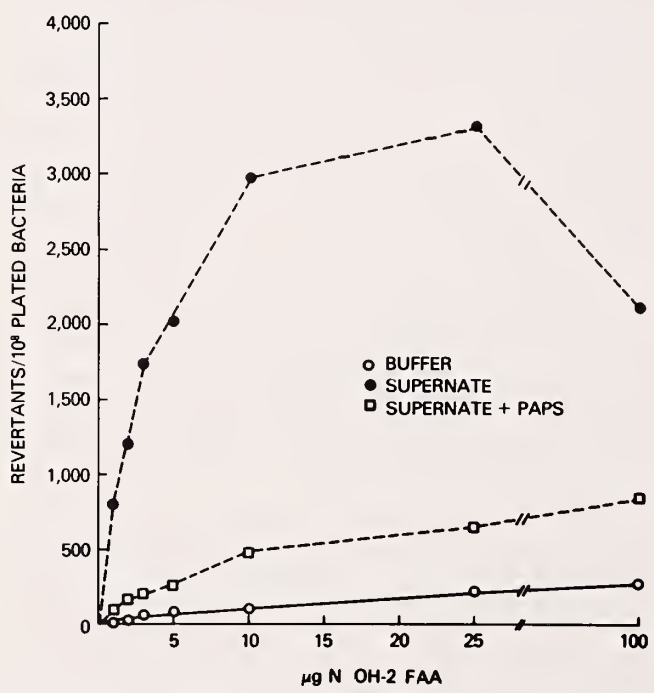
[a] Data are from (*22*). Bacterial tester strain TA1538 was used with 3 μg *N*-OH-2-FAA.

[b] Values represent No. of revertants.

TABLE 2.—*Effect of sulfation on deacetylation and covalent binding of* N-*OH-2-FAA*[a]

Conditions	Revertants/10^8 plated bacteria	Acetate, nmol	Covalent binding, nmol
Buffer	65	0	0
Supernatant	1,750	1.5	0.2
Supernatant + ascorbic acid	2,896	2.3	0.2
Supernatant + PAPS	252	0	0.6
Supernatant + PAPS + ascorbic acid	2,910	0	0.1

[a] Data are from (*22*). Mutagenesis data are from table 1; for the deacetylation experiment, 16 nmol *N*-OH-2-FAA was used. Ascorbic acid concentration was 1.9 mM.



TEXT-FIGURE 1.—Effect of supernatant and supernatant plus PAPS on mutagenesis of *N*-OH-2-FAA. Data are from (*22*).

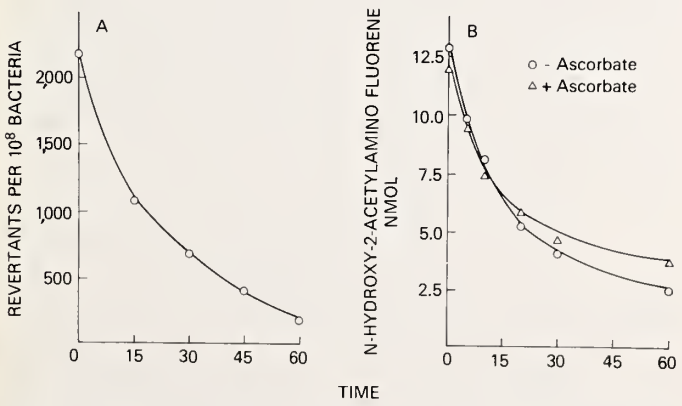
TABLE 3.—*Metabolism of [^{14}C acetyl]N-OH-2-FAA under conditions for sulfation*[a]

Metabolites	Supernatant	Supernatant + ascorbic acid	Supernatant + PAPS	Supernatant + PAPS + ascorbate
2-FAA	4.5	12.5	11.2	62.4
Acetic acid	9.4	14.1	0	0
Covalent binding	0.1	0.1	4.0	0.7
Others	0	0	60.7	13.2

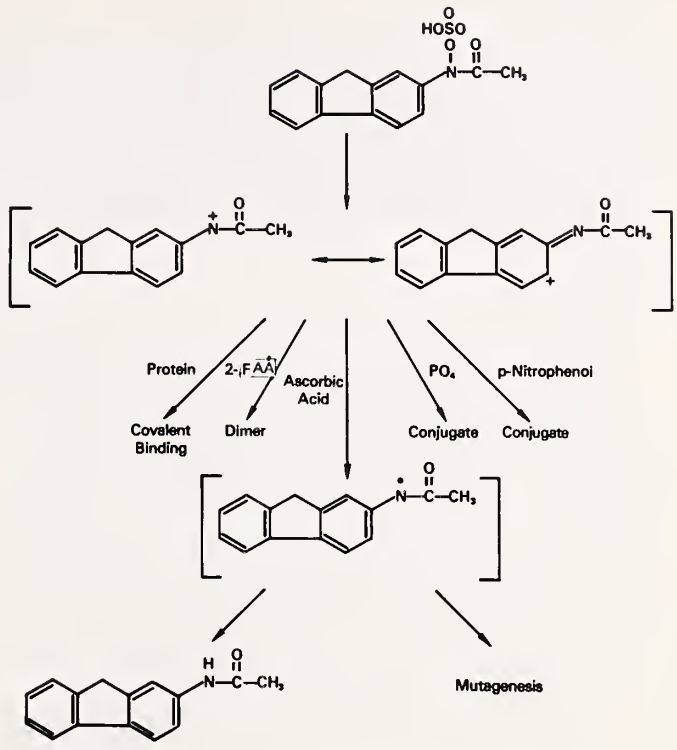
[a] Data, from (*22*), are expressed as percentage metabolism of 16 nmol *N*-OH-2-FAA. The other metabolites isolated were: a dimer of 2-FAA, a *p*-nitrophenol adduct, and a phosphate adduct of 7-OH-2-FAA.

tion mixture containing [^{14}C acetyl]*N*-OH-2-FAA, we observed approximately a sixfold increase in the formation of 2-FAA at the expense of the other metabolites (table 3), but we found no evidence that *N*-OH-2-FA was formed because acetic acid was not a detectable metabolite. Thus ascorbic acid apparently reduced the reactive metabolite to 2-FAA, which is nonmutagenic, and thereby inhibited its reaction with various nucleophiles in the incubation mixture (*22*).

The potential role of an unstable metabolite that did not lead to covalent binding was subsequently investigated by the addition of the bacteria at various times to an incubation mixture containing sulfotransferase, the PAPS generating system, *N*-OH-2-FAA, and ascorbic acid. Under similar conditions, the disappearance of [^{14}C acetyl]-*N*-OH-2-FAA from the incubation mixture was studied (text-fig. 2). The similarity of the curves indicate that formation of the mutagenic metabolite was apparently mediated by the sulfation of *N*-OH-2-FAA and that the metabolite did not accumulate in the incubation mixture (*22*).



TEXT-FIGURE 2.—A) Time course of *N*-OH-2-FAA mutagenesis under conditions for sulfation. The bacteria were added at the indicated time. Data are from (*22*). B) Effect of ascorbic acid on metabolism of *N*-OH-2-FAA under conditions for sulfation. Data are from (*22*).



TEXT-FIGURE 3.—Proposed mechanism for conversion of *N*-2-FAA-sulfate to mutagenic metabolite.

Inasmuch as a reduction of the nitrenium ion of 2-FAA presumably would occur by two successive one-electron additions (*23, 24*), it seems reasonable that the mutagenic metabolite is a free radical of 2-FAA, which is illustrated in text-figure 3 (*22*).

The mechanism by which the proposed 2-FAA free radical might cause the carcinogenesis of the parent compound is unknown. DeBaun et al. (*7*) presented evidence that sulfation of *N*-OH-2-FAA may be an important step in its hepatocarcinogenesis. Because the free radical is formed from the same reactive metabolite that covalently binds to protein or DNA, it cannot be determined at this time whether the hepatocarcinogenesis is mediated by covalent binding or by the free radical (*22, 26*). However, that the concentrations of ascorbic acid and NADPH used in the mutagenesis study are similar to those present in the liver is worthy of attention.

If a free radical of 2-FAA formed by its sulfation is important in the hepatocarcinogenesis of *N*-OH-2-FAA, other radicals produced from it may also be mutagenic or carcinogenic (*26*). For example, it is well-known that hydroxylamines can be easily oxidized (*27*). Inasmuch as *N*-OH-2-FA is mutagenic in the Ames' bacterial test system (*17*), this mutagenicity may be partly mediated by oxidation to a nitroxide radical (*26*). Also, at least 2 other radicals may potentially be formed from *N*-OH-2-FAA; these are its nitroxide (*28, 29*) and 2-FA radicals (*26*). Their possible roles in mutagenicity or carcinogenicity are unknown.

APPENDIX

After the presentation of this paper, Wirth and Thorgeirsson (*30*) have obtained evidence that ascorbic acid increases the mutagenic activity of *N*-OH-2-FAA in the presence of a purified *trans* acetylase but not in that of a purified sulfotransferase. Thus the stimulatory effects of ascorbic acid do not appear to be due to the proposed free radical of 2-FAA.

REFERENCES

(*1*) Weisburger JH, Weisburger EK: Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1–66, 1973

(*2*) Miller JA: Carcinogenesis by chemicals: An overview. G.H.A. Clowes Memorial Lecture. Cancer Res 30: 559–576, 1970

(*3*) Miller EC: Some current perspectives on chemical carcinogenesis in humans and experimental animals. Presidential address. Cancer Res 38:1479–1496, 1978

(*4*) Cramer JW, Miller JA, Miller EC: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J Biol Chem 235:885–888, 1960

(*5*) Miller EC, Miller JA, Hartmann HA: *N*-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res 21:815–824, 1961

(*6*) Thorgeirsson SS, Jollow DJ, Sasame HA, et al: The role of cytochrome P_{450} in N-hydroxylation of 2-acetylaminofluorene. Mol Pharmacol 9:398–404, 1973

(*7*) DeBaun JR, Miller EC, Miller JA: *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and its protein-(methion-*S*-yl) binding in rat liver. Cancer Res 30:577–595, 1970

(*8*) King CM, Phillips B: Enzyme-catalyzed reactions of the carcinogen *N*-hydroxy-2-fluorenylacetamide with nucleic acids. Science 159:1351–1353, 1968

(*9*) Irving CC, Janss DH, Russell LT: Lack of hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and Zymbal's gland of the rat. Cancer Res 31:387–391, 1971

(*10*) Bartsch H, Dworkin C, Miller EC, et al: Formation of electrophilic N-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-4-acetylaminobiphenyl. Biochim Biophys Acta 304:42–55, 1973

(*11*) King CM, Allaben WT: The role of arylhydroxamic acid *N*-*O*-acyltransferase in the carcinogenicity of aromatic amines. *In* Conjugation Reactions in Drug Biotransformation (Aitio A, ed). Amsterdam: Elsevier, 1978, pp 431–441

(*12*) Ames BN, McCann J, Yamasaki E: Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat Res 31: 347–364, 1975

(*13*) McCann J, Choi E, Yamasaki E, et al: Detection of carcinogens in the *Salmonella*/microsome test: Assay of 300 chemicals. Proc Natl Acad Sci USA 72:5135–5139, 1975

(*14*) McCann J, Ames BN: Detection of carcinogens as mutagens in the *Salmonella*/microsome test; assay of 300 chemicals: Discussion. Proc Natl Acad Sci USA 73:950–954, 1976

(*15*) Mulder GJ, Hinson JA, Gillette JR: Generation of reactive metabolites of *N*-hydroxyphenacetin by glucuronidation and sulfation. Biochem Pharmacol 26:189–196, 1977

(*16*) _____: Conversion of the *N*-*O*-glucuronide and *N*-*O*-sulfate conjugates of *N*-hydroxyphenacetin to reactive intermediates. Biochem Pharmacol 27:1641–1649, 1978

(*17*) Durston EW, Ames BN: A simple method for the detection of mutagens in urine: Studies with the carcinogen 2-acetylaminofluorene. Proc Natl Acad Sci USA 71:737–741, 1974

(*18*) Weeks CE, Allaben WT, Louie SC, et al: Role of arylhydroxamic acid acyltransferase in the mutagenicity of *N*-hydroxy-*N*-2-fluorenylacetamide in *Salmonella typhimurium*. Cancer Res 38:613–618, 1978

(*19*) Sakai S, Reinhold CE, Wirth PJ, et al: Mechanism of in vitro mutagenic activation and covalent binding of *N*-hydroxy-2-acetylaminofluorene in isolated liver cell nuclei from rat and mouse. Cancer Res 38:2058–2067, 1978

(*20*) Stout DL, Baptist JN, Matney TS, et al: *N*-Hydroxy-2-aminofluorene: The principal mutagen produced from *N*-hydroxy-2-acetylaminofluorene by a mammalian supernatant enzyme preparation. Cancer Lett 1: 269-274, 1976

(*21*) Mulder GJ, Hinson JA, Nelson WL, et al: Role of sulfotransferase from rat liver in the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in *Salmonella typhimurium*. Biochem Pharmacol 26:1356–1358, 1977

(*22*) Andrews LS, Hinson JA, Gillette JR: Studies on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in the Ames-*Salmonella* mutagenesis test system. Biochem Pharmacol 27:2399–2408, 1978

(*23*) Scribner JD, Naimy NR: Destruction of triplet nitrenium ion by ascorbic acid. Experientia 31:470–471, 1975

(*24*) _____: Reaction of esters of *N*-hydroxy-2-acetamidophenanthrene with cellular nucleophiles and the formation of free radicals upon decomposition of *N*-acetoxy-*N*-arylacetamides. Cancer Res 33:1159–1164, 1973

(*25*) Andrews LS, Fysh JM, Hinson JA, et al: Ascorbic acid inhibits covalent binding of enzymatically generated 2-acetyl-aminofluorene-*N*-sulfate to DNA under conditions in which it increases mutagenesis in *Salmonella* TA1538. Life Sci 24:59–64, 1979

(*26*) Hinson JA, Andrews LS, Gillette JR: A possible role of free radicals in the mutagenicity of *N*-hydroxy-2-acetylaminofluorene. *In* Free Radicals and Cancer (Floyd R, ed). New York: Marcel Dekker, 1981. In press

(*27*) Sidgwich NV, Millar IT, Springall HD: Sidgwich's Organic Chemistry of Nitrogen. Oxford: Clarendon Press, 1966, p 306

(*28*) Bartsch H, Hecher E: On the metabolic activation of the carcinogen *N*-hydroxy-*N*-acetylaminofluorene. III. Oxidation with horseradish peroxidase to yield 2-nitrosofluorene and *N*-acetoxy-*N*-acetylaminofluorene. Biochim Biophys Acta 237:567–578, 1971

(*29*) Floyd RA, Soong LM: Obligatory free radical intermediate in the oxidative activation of the carcinogen *N*-hydroxy-2-acetylaminofluorene. Biochim Biophys Acta 498:244–249, 1977

(*30*) Wirth PJ, Thorgeirsson SS: Mechanism of *N*-hydroxy-2-*S*-acetylaminofluorene mutagenicity in the *Salmonella* test system: Role of *N*-O-acyltransferase and sulfotransferase from rat liver. Mol Pharmacol 19:337–344, 1981

Formation, Metabolic Activation by N,O-Acyltransfer, and Hydrolysis of *N*-Acyl-*N*-arylamine Derivatives[1, 2]

Charles M. King[3] *and* Wendell W. Weber[4]

ABSTRACT—Amines and hydroxylamines can be produced from N-substituted aryl compounds by many organisms. Both functional groups can be N-acetylated enzymatically by preparations from the tissues of most mammalian species. The N-acylated products are subject to further metabolism including oxidation, hydrolysis, conjugation, or, with arylhydroxamic acids, N-O-acyltransfer. These reactions are of concern because they lead to changes in the levels of compounds that can alter tissue macromolecules. Select *N*-hydroxyarylamines and *N*-acyloxyarylamines can react with nucleic acid, and the hydroxylamines are mutagenic in bacterial systems. Thus the relative rates of these biochemical reactions within an organism may determine whether exposure to an arylamine will cause an adverse biologic effect. The ability of an arylamine to induce tumors reflects the composite effects of genetic determinants (i.e., species, strain, sex, and organ) as well as the exposure to xenobiotic compounds because these factors modulate the metabolic pathways crucial to the carcinogenic process. The extreme genetic differences in the levels of the enzymes involved in the metabolism of N-acyl derivatives of arylamines in man and experimental animals offers an experimental approach that may aid in elucidating the mechanisms by which these compounds induce cancer.—Natl Cancer Inst Monogr 58: 117–122, 1981.

The adverse biologic properties of both arylamines and arylnitro derivatives are believed to be derived from their biochemical conversion to metabolites of intermediate oxidation states as described elsewhere in this volume. An important factor in the expression of the mutagenic, carcinogenic, and more acute effects of these compounds is that of acetylation. Most mammalian species are capable of forming and hydrolyzing N-acetyl derivatives. The capacity of an organism to perform these reactions can indirectly influence whether an exposure will lead to a toxic response by determinination of the levels of free or acetylated metabolites that mediate the deleterious effects of these compounds. However, acetyl transfer can also be directly responsible for the harmful effects of arylamine derivatives as seen in the production of reactive *N*-acetoxyarylamines from arylhydroxamic acids by N,O-acyltransfer. Thus the formation and metabolism of acetylated metabolites must be considered if one is to attempt to evaluate the effects of arylamine derivatives in biologic systems.

ACETYLATION

Acetylation is a common route of arylamine biotransformation in many animals species including man (text-fig. 1). Studies in humans and in various intact animals show large species differences in the capacity for excretion of acetylated arylamines. For example, the rabbit has a high capacity to acetylate arylamine drugs such as sulfanilamide in the N-4 position. Man has a moderate capacity for this process, whereas other species, such as the dog and the fox, are apparently unable to acetylate sulfanilamide in that position. The latter two species can acetylate the sulfonamide group and excrete N^1-acetylsulfanilamide (*1*).

The capacity for N-acetylation appears essential in the modulation of the occurrence of arylamine-induced tumors in selected organs and the susceptibility of different tissues to arylamine carcinogens. The unique case in point is the dog, which develops tumors of both liver and urinary bladder with chronic administration of acetylated arylamines, whereas it develops only urinary bladder tumors when the unacetylated parent compound is given. This is especially interesting, inasmuch as the dog is one species incapable of N-acetylating any of the known arylamine carcinogens (*2, 3*). Because the arylacetamides and their corresponding arylamines are N-hydroxylated in the dog, Poirier et al. (*3*) proposed that 1 of the proximate carcinogens in the urinary bladder might be a hydroxylamine, whereas in liver, an *N*-acetylhydroxylamine (arylhydroxamic acid) might be required for carcinogenesis. They were careful to point out, however, that these differences in carcinogenicity might reside in differences in metabolic pathways or rates of metabolism in liver and bladder and that the reactive carcinogen responsible for tumor induction might be the same for both tissues.

One aspect of N-acetylation in man and rabbit that particularly attracts attention is the existence of a hereditary polymorphism in the rate of drug acetylation (*1, 4*).

Abbreviations: 2-FA = 2-fluorenamine; *N*-OH-2-FAA = *N*-hydroxy-2-fluorenylacetamide.

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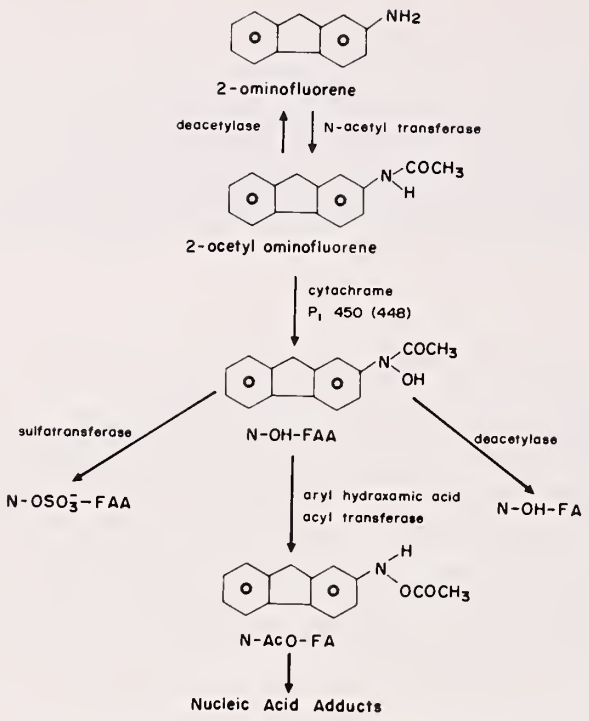

TEXT-FIGURE 1.—Metabolic activation of 2-FA.

Individuals can be classified as rapid or slow acetylators of drugs such as isoniazid and sulfamethazine. This acetylation polymorphism is transmitted as an autosomal single gene trait in which slow acetylators are homozygous for a recessive allele (*rr*) and rapid acetylators are either homozygous (*RR*) or heterozygous (*Rr*) for a dominant allele. After the administration of a drug such as isoniazid, circulating levels are usually several times higher for any specified period in slow than in rapid acetylators. Consequently, slow acetylators accumulate high concentrations of isoniazid and other polymorphically acetylated drugs with prolonged intake of ordinary doses of the drugs and are more inclined to develop drug toxicities (*4*). Association of rapid acetylation with specific drug toxicities is the subject of controversy at the present time. Mitchell et al. (*5*) reported strong evidence that rapid acetylators are more susceptible to isoniazid-induced liver damage, and animal studies have shown that the metabolite monoacetylhydrazine, which is produced more quickly in rapid acetylators, can be biotransformed by liver microsomal enzymes to a potent acylating agent capable of causing liver necrosis. Investigations by others indicated that rapid acetylators may not be more prone to isoniazid-induced liver damage than are the slow ones (*6*). Evidence has also been presented that monoacetylhydrazine is not only produced faster by rapid acetylators but is also eliminated earlier by conversion to the nontoxic diacetylhydrazine derivative (*7, 8*). Undoubtedly, the acetylation polymorphism is significant in the determination of the outcome of chronic toxicities with these drugs. Knowledge of this hereditary trait has advanced our understanding of the importance of individual differences in acetylating capacity in regard to these effects, although further basic studies are needed before the toxic mechanisms involved can be clarified.

Studies on human and rabbit tissues have shown that the hereditary differences in N-acetylation are attributable to a polymorphism of an acetyl coenzyme A-dependent *N*-acetyltransferase (E.C. 2.3.1.5) located in liver and small intestinal mucosa that catalyzes the acetylation of amino and hydrazino groups to form amides (*1, 4*). Recently, Glowinski et al. (*9*) demonstrated that the arylamines: aminofluorene, α-naphthylamine, β-naphthylamine, benzidine, and methylene-bis-2-chloroaniline are acetylated by the same polymorphic *N*-acetyltransferase as isoniazid and sulfamethazine in human and rabbit tissues (table 1). The fact that these compounds are substrates for polymorphic *N*-acetyltransferase raises the possibility that the acetylator phenotype may confer differences in individual susceptibility to arylamine carcinogenesis.

Closely related to this finding are the studies of Lower et al. (*10*) who suggested a slightly higher incidence of slow acetylators among patients with bladder cancer. However, clarification of the role of acetylation in the induction of arylamine-induced bladder cancer in man awaits the results of studies on a population of patients whose bladder tumors resulted from aromatic amine exposure and who are compared with similarly exposed but disease-free controls (*10*). An alternate approach to the question of *N*-acetyltransferase phenotype and its relationship to aromatic amine carcinogenesis is through the use of experimental animals. One should also note that acetyl transfer is involved in two steps of the arylamine activation pathway, i.e., in the formation of amides from amines through the polymorphic acetyl coenzyme A-dependent *N*-acetyltransferase and in the generation of reactive *N*-acetoxyarylamines from *N*-hydroxyarylacetamides by the action of arylhydroxamic acid acyltransferase. These two enzymes have highly similar properties and are also under common genetic control in rabbit liver (*11, 12*). The latter observation adds further indirect support to the possibility that rapid and slow isoniazid acetylators differ in respect to susceptibility to carcinogenicity from exposure to arylamines.

Because of marked species variation in metabolic processes, no one species can serve as a model for the biotransformation of any foreign compound in all humans, nor has any single species been found to mimic exactly the metabolism of any foreign compound in any individual (*13*). Similarly, the induction of tumors by an aromatic amine in the same organ of different species or in the various organs of the same individual does not necessarily result from the same metabolic pathway.

The occurrence of different *N*-acetyltransferases in different species has posed a problem for researchers in the selection of appropriate animals in which to evaluate mechanisms of toxicity from arylamines for man. The dog (and possibly also the fox) appears to represent a species comprised entirely of genetically slow acetylators that has been used to advantage for this purpose. Mice and rats have been used extensively and rabbits less so to discern the effects of these agents in intact animals. The existence of a hereditary acetylator polymorphism which confers large predictable differences on the capacity of individuals to

TABLE 1.—*N-Acetyltransferase activity in the livers of rapid and slow acetylators among humans and rabbits*[a]

Compounds	Rabbit[b]			Human		
	Activity, nmol/min/mg		Ratio	Activity, nmol/min/mg		Ratio
	Rapid	Slow		Rapid	Slow	
Test drugs						
Sulfamethazine	6.4	0.037	173	0.84	0.23	3.7
p-Aminobenzoic acid	1.2	0.50	2.4	0.15	0.16	0.94
Arylamine carcinogens						
α-Naphthylamine	12.4	0.023	540	1.2	0.23	5.2
Benzidine	6.5	0.019	315	0.17	0.019	8.9
β-Naphthylamine	8.7	0.028	310	0.23	0.026	8.8
2-FA	7.5	0.013	580	0.28	0.021	13
Methylene-bis-2-chloroaniline	—	—	—	0.14	0.015	9.3
Mean			436			9.0

[a] *See* (*9*).
[b] Dashes = not determined.

N-acetylate arylamine carcinogens has been thoroughly studied in the rabbit. The occurrence of such differences involving N-acetyl transfer within a single species such as the rabbit would seem to offer an advantage over the dog in our obtaining further insight into the importance of N-acetylation steps in the metabolic activation of arylamines to carcinogens, particularly because the experimental induction of tumors of the urinary bladder and other tissues by systemically administered arylamines and related compounds has been demonstrated in the rabbit (*14, 15*). Only recently have studies of inbred mouse strains shown that A/J strain mice are slow acetylators of aminofluorene and benzidine compared with C57BL/6J mice and approximately 15 other strains surveyed [(*4*); Glowinski IB, Weber WW: Unpublished observations]. Inbred mouse strains which exhibit variation in N-acetylation capacity as well as in other reactions in the activation pathway would appear to be other useful models for the study of mechanisms of arylamine-induced cancer, particularly to facilitate genetic analysis of the tumorigenic process (*16*). The full potential of these animals as models for such investigations is at this time largely unexplored.

ACTIVATION OF ARYLHYDROXAMIC ACIDS BY N,O-ACYLTRANSFER

The arylamine-substituted nucleic acids formed on incubation of the arylhydroxamic acids with tRNA and rat liver cytosol (*17, 18*) resulted from the formation of reactive *N*-acetoxyarylamines as a consequence of N,O-acyltransfer (*19–21*), which is illustrated in text-figure 1. The role of this metabolic pathway in the carcinogenicity of arylamines has been reviewed recently (*22, 23*).

Whereas the reactivity of the metabolites responsible for the formation of these adducts has precluded both their isolation and synthesis, indirect evidence for the N,O-acyltransfer mechanism has come from the recognition that 1) the enzymes in rat (*21*) and rabbit liver (Fysh JM, Vaught JB, Glowinski IB, et al: Unpublished observations) responsible for the activation of the arylhydroxamic acids could not be resolved from the enzyme that transfers the acetyl moiety of the hydroxamic acid to other arylamines, 2) the tissue distribution of these enzymes in the rat were similar (*21*), 3) methylation of the oxygen of the hydroxamic acids blocked adduct formation but did not prevent acyltransfer to arylamines (*21*), and 4) arylamines decreased adduct formation of the hydroxamic acids with nucleic acids (*21*).

Thus far studies with N-acetylated substrates have indicated that the arylhydroxamic acid acyltransferases are found in the supernatants of cell homogenates. The enzyme from rat liver migrates on gel filtration as a polypeptide with a molecular weight of 28,000 (*21*); the rabbit liver enzyme has a molecular weight of 33,000 in a sodium dodecylsulfate-polyacrylamide electrophoresis system (*12*). Although the rat and rabbit enzymes are inhibited by compounds capable of reaction with sulfhydryl groups (*4, 21*) and are protected by dithiothreitol (*12, 21*), rat liver acyltransferase is not inhibited by the microsomal arylamidase inhibitor diethyl *p*-nitrophenyl phosphate (*24*). Both the rat and rabbit liver enzymes have isoelectric points of approximately 5 (*4, 23*); the purification scheme used successfully for these enzymes consisted of sequential centrifugation of homogenates at 105,000×*g*, fractional precipitation with ammonium sulfate, ion-exchange chromatography on DEAE cellulose, gel filtration, and electrophoresis on polyacrylamide gels (*12, 23*). Protection against activity losses due to oxidation was achieved by use of argon atmospheres and the incorporation of dithiothreitol in all buffers. Detailed studies of the rabbit liver enzyme have shown it to be indistinguishable, genetically and chemically, from the acetyl coenzyme A-dependent *N*-acetyltransferase of this tissue (*11, 12*).

Arylhydroxamic acid acyltransferase is widely distributed. When using *N*-OH-2-FAA as substrate, we observed activity in one or more tissues from 9 species, including the human; only tissues of the dog and the goat failed to yield detectable levels of the enzyme (table 2). With the important exceptions of the urinary bladders of man and the dog, the target organs of aromatic amines in these species, arylhydroxamic acid acyltransferase was in most susceptible tissues that have been studied as well as in tissues that normally do not develop tumors when these carcinogens are administered. The highest acyltransferase activities are usually found in the liver, with lesser quantities in other organs; the activities are independent of the sex of the animal.

TABLE 2.—*Relative arylhydroxamic acid acyltransferase activities in tissues from various species*[a]

Tissue	Rat	Hamster	Rabbit	Guinea pig	Monkey	Baboon	Pig	Human	Mouse	Dog and goat
Liver	111[b, c]	278[b]	371	9	56	58	32	12	5	2[b]
Kidney	29	11	4	12					<2	<2
Small intestine	36[b, d]	118[b, d]	43	12[b]	20			17	<2	<2
Colon	38	31	6	10	<2			5	<2	<2
Stomach	24[b]	36[b]	2	14	<2				<2	<2
Lung	13	18	3	3	<2			2	<2	
Mammary gland	10[b, c]									
Zymbal's gland	10[b]									
Spleen	7	4	2	3	<2				<2	<2
Brain	3	6	2	4						
Uterus			10[b]							
Bladder			20[b]							

[a] Activity was measured as 10^{-11} mol of 2-FA bound to tRNA when incubated with cytosol. Activities were assayed with the use of cytosols, tRNA as a trapping agent, and *N*-OH-2-FAA (*21*). Data were compiled from previous publications (*21, 27–30*) and from the authors' unpublished data.

[b] Tissues are those in which 2-FA derivatives most commonly induce tumors.

[c] Cytosols contain only 1 acyltransferase as determined by gel filtration with *N*-OH-2-FAA as substrate.

[d] Tissues contain 2 acyltransferases that are separable by gel filtration as determined with *N*-OH-2-FAA.

Arylhydroxamic acid acyltransferases are capable of using a wide range of monocyclic, dicyclic, and tricyclic substrates (*23*), and nucleic acid adducts were formed in experiments with formyl, acetyl, and propionyl derivatives (*25*). However, the *O*-glucuronide of *N*-OH-2-FAA did not serve as a substrate (*26*).

Multiple forms of arylhydroxamic acid acyltransferases exist; two species of acyltransferase have been demonstrated in the small intestine of the rat and in the small intestine and liver of the hamster as judged by molecular weight, immunologic characteristics, and/or aryl moiety specificity (*23*). Evidence is now accumulating that similar complexities can be detected with the use of various acyl groups in preparations from human (*27*), rat (Fysh JM, King CM: Unpublished observations), and rabbit tissues (Fysh JM, Vaught JB, Glowinski IB, et al: Unpublished observations).

An important consequence of the metabolism of arylhydroxamic acids by these acyltransferases is that the products can react covalently with either proteins or nucleic acids. The nucleic acid modifications in vitro result in the introduction of arylamine substituents at the C-8 position of guanine residues (*31*) and possibly at other nucleic acid sites (*32, 33*). The instability of the adducts can lead to the loss of the arylamine moiety without restoration of the guanine content of the nucleic acid (*32*), and, with RNA, it can result in cleavage of the polynucleotide chain and release of a nonpolar carcinogen derivative (*33*). Nucleic acid adducts formed in vivo on administration of-*N*-OH-2-FAA (*28, 34–36*) or *N*-OH-4-acetylaminobiphenyl (*37*) to rats are compatible with acyltransferase-catalyzed metabolic activation of these compounds, i.e., the adducts have been formed with loss of the acetyl moiety.

Arylhydroxamic acid acyltransferase can serve as a metabolic activation system for its substrates in the *Salmonella* mutagenicity system (*24, 25*). Unexpectedly, the mutations do not arise from the reactive *N*-acetoxyarylamine products. Structure activity studies in which this technique is used have shown that the ability of acyltransferase to catalyze the formation of mutagens from substrates is not related to the enzyme's ability to produce substances that can react spontaneously with nucleic acid. Rather, these observations have been interpreted as having resulted from the hydrolytic production of arylhydroxylamines and a subsequent terminal metabolic activation of the hydroxylamine within the bacterial cell (*24, 25*).

Evidence for the participation of a reactive metabolite in carcinogenesis can come from several experimental approaches, including 1) the tissue distribution of enzymes capable of generating the reactive species, 2) evidence of the effectiveness of the enzyme in the target organ as judged by the generation of nucleic acid adducts in vivo, and 3) studies of the relative carcinogenicities of structurally similar substrates. Application of these methods in the rat demonstrated that both liver and mammary gland carcinogenesis in this species may depend on the metabolic activation of arylhydroxamic acids by N,O-acyltransfer.

The liver and the mammary gland of the rat possess acyltransferases capable of metabolizing arylhydroxamic acids to species that can react with nucleic acids. Adducts formed with liver DNA (*31, 32*) and with rRNA of the mammary gland (*36*) when rats receive arylhydroxamic acids are consistent with activation of these compounds by acyltransfer, i.e., adduct formation occurs without retention of the acetyl moiety.

Liver tumor induction in the rat appears to be potentiated by the hepatotoxicity of the carcinogen. Thus the nonhepatotoxic *N*-OH-4-acetylaminobiphenyl does not induce liver tumors even though aminobiphenyl-substituted DNA is formed to about the same extent as adducts with the toxic and carcinogenic *N*-OH-2-FAA (*37*), the carcinogenicity of which can be drastically reduced by the administration of *p*-hydroxyacetanilide, an inhibitor of acyltransferase (*38*). Furthermore, the carcinogenicity of *N*-OH-2-FAA was not fully restored with the simultaneous administration of the phenol and excess sulfate, which should have favored the conjugation of the hydroxamic acid with sulfate, the metabolic activation pathway most often associated with the hepatocarcinogenicity of arylhydroxamic acids in the rat. Therefore, the apparent lack of correlation between the acyltransferase-catalyzed activation and hepatocarcinogenicity of arylhydroxamic acids

may not come from the inability of the compounds to alter the genome of the cell but rather from their failure to fix this lesion by inducing replication of the modified DNA as a consequence of hepatotoxicity.

Many aromatic amines can induce tumors of the rat mammary gland as discussed elsewhere in this volume. Tumors can be induced by the direct application of *N*-OH-2-FAA, but not by application of its hydrolysis product *N*-OH-2-FA nor by its reduction product 2-FAA (*39*). Comparison of fluorenylhydroxamic (*40*) and biphenylhydroxamic acids (Shirai T, Fysh JM, Lee MS, et al: Unpublished observations) that possess N-formyl, N-acetyl, or N-propionyl moieties have indicated that the ability of these compounds to induce mammary gland tumors is related to their ability to yield nucleic acid adducts in vitro when they are incubated with partially purified acyltransferase preparations from either the mammary gland or liver; the acetylated derivatives were most active. The activation of arylhydroxamic acids by acyltransfer is the only metabolic pathway of the mammary gland that has been demonstrated to catalyze the formation of nucleic acid adducts with aromatic amine derivatives and could account for the induction of mammary tumors by these compounds.

DEACETYLATION OF N-ACYLARYLAMINE DERIVATIVES

Enzymes present in many species can hydrolyze the N-acyl groups of arylhydroxamic acids and arylacylamides (text-figure 1). Typically, these reactions have been followed by determination of the release of the acyl moiety and/or the increase in arylamine or arylhydroxylamine (*41, 42*). Such studies have been facilitated with radiochemical-labeled acyl moieties and agents that can complex and stabilize the unstable hydroxylamines released in the reaction.

The acylated arylamine derivatives that attract attention because of their potentially adverse toxic properties are usually hydrolyzed most readily by microsomal enzymes (*41, 42*). These deacylases are inhibited by reagents capable of reaction with sulfhydryl groups and by the esterase inhibitor diethyl *p*-nitrophenyl phosphate (*41, 42*). Two deacylases with molecular weights of 41,000 and 200,000 have been isolated from the microsomes of guinea pig liver (*43*). The purification procedures used sequentially included solubilization by ultrasound, fractional precipitation with ammonium sulfate, ion-exchange chromatography on DEAE-cellulose, chromatography on hydroxyapatite, and a second ion-exchange chromatographic step.

Generally, the levels of deacylase are highest in the guinea pig; other mammalian species tested have measurable levels of activity (*41, 42*). Although the liver of most species usually contained higher deacylase activity than other tissues, that of the Zymbal gland of the rat was even greater (*43*).

The enzymatic hydrolysis of amides is facilitated by acyl groups that are good "leaving" groups; amides containing *N*-formyl, monochloroacetyl, or trifluoroacetyl moieties are more easily hydrolyzed than the comparable acetyl derivatives (*42*). Both the aromatic nucleus and the position of the amide substituents affect the rate at which the compounds will be hydrolyzed (*42*). Guinea pig microsomes hydrolyze *N*-OH-2-FAA more readily than 2-FAA (*41, 42*); the N-acetyl moiety of the *O*-glucuronide of the hydroxamic acid can be hydrolzyed to yield the *O*-glucuronide of the arylhydroxylamine which can react with C-8 of guanine-containing molecules (*26*).

Although hydrolysis of arylhydroxamic acids yields arylhydroxylamines that react with nucleic acids in acidic media, such reactions are of limited importance at neutrality and in buffers at physiologic pH. However, the deacetylation of arylhydroxamic acids can affect the development of mutagenic arylhydroxylamines in the *Salmonella typhimurium* mutagenicity systems (*44*), and the arylhydroxylamines may also react with protein to exert adverse biologic effects.

Probably, the role of deacetylation in carcinogenesis is indirect. For example, deacetylation tends to increase tumorigenesis when arylhydroxylamines are involved, as in the urinary bladder of the dog, and to decrease cancer incidence when arylhydroxamic acids are involved, as in the liver and mammary gland of the rat.

REFERENCES

(*1*) Weber WW: Acetylation of drugs. *In* Metabolic Conjugation and Metabolic Hydrolysis (Fishman WH, ed), vol 3. New York: Academic Press, 1973, pp 249–296

(*2*) Lower GM Jr, Bryan GT: Enzymatic *N*-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. Biochem Pharmacol 22:1581–1588, 1973

(*3*) Poirier LA, Miller JA, Miller EC: The *N*- and ring-hydroxylation of 2-acetylaminofluorene and the failure to detect *N*-acetylation of 2-aminofluorene in the dog. Cancer Res 23:790–800, 1963

(*4*) Weber WW, Glowinski IB: Acetylation. *In* Enzymatic Basis of Detoxication (Jakoby WB, ed). New York: Academic Press, 1979, pp 169–186

(*5*) Mitchell JR, Zimmerman HJ, Ishak KG, et al: Isoniazid liver injury: Clinical spectrum, pathology and probable pathogenesis. Ann Intern Med 84:181–192, 1976

(*6*) Ellard GA, Mitcheson DA, Girling DJ, et al: The hepatic toxicity of isonizaid among rapid and slow acetylators of the drug. Am Rev Respir Dis 118:628–629, 1978

(*7*) Ellard GA, Gammon PT: Pharmacokinetics of isoniazid metabolism in man. J Pharmacokinet Biopharm 4: 83-112, 1976

(*8*) Timbrell JA, Wright JM, Baillie TA: Monoacetyl hydrazine as a metabolite of isoniazid in man. Clin Pharmacol Ther 22:602–608, 1977

(*9*) Glowinski IB, Radtke HE, Weber WW: Genetic variation in *N*-acetylation of carcinogenic arylamines by human and rabbit liver. Mol Pharmacol 14:940–949, 1978

(*10*) Lower GM Jr, Nilsson T, Nelson CE, et al: *N*-Acetyltransferase phenotype and risk in urinary bladder cancer: Approaches in molecular epidemiology. Preliminary results in Sweden and Denmark. Environ Health Perspect 29:71–79, 1979

(*11*) Glowinski IB, Weber WW, Fysh JM, et al: Metabolism of arylamines: Evidence for the identity of arylhydroxamic

acid acyltransferase (AHAT) and genetically polymorphic *N*-acetyltransferase (NAT) of rabbit liver. Proc Am Assoc Cancer Res 20:117, 1979

(*12*) GLOWINSKI IB, FYSH JM, VAUGHT JB, et al: Evidence that arylhydroxamic acid *N,O*-acyltransferase and the genetically polymorphic *N*-acetyltransferase are properties of the same enzyme in rabbit liver. J Biol Chem 225: 7883–7890, 1980

(*13*) GILLETTE JR: Comparative metabolism and the choice of experimental animals. *In* 5th ICLA Symposium (Fischer G, ed). Stuttgart: Gustav Fischer Verlag, 1973, pp 1–15

(*14*) IRVING CC, WISEMAN R JR, YOUNG JM: Carcinogenicity of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene in the rabbit. Cancer Res 29:838–848, 1967

(*15*) WOOD M: Aetiology of tumours of the urinary bladder. Induction of tumours in the urinary tract of the rabbit by aromatic amines. Ind Med Surg 39:55–61, 1970

(*16*) TANNEN RW, WEBER WW: Rodent models of the human isoniazid-acetylator polymorphism. Drug Metab Dispos 7274–7279, 1979

(*17*) KING CM, PHILLIPS B: Enzyme-catalyzed reactions of the carcinogen *N*-hydroxy-2-fluorenylacetamide with nucleic acid. Science 159:1351–1353, 1968

(*18*) DEBAUN JR, ROWLEY JY, MILLER EC, et al: Sulfotransferase activation of *N*-hydroxy-2-acetylaminofluorene in rodent livers susceptible and resistant to this carcinogen. Proc Soc Exp Biol Med 129:268–273, 1968

(*19*) BARTSCH H, DWORKIN M, MILLER JA, et al: Electrophilic *N*-acetoxyaminoarenes derived from carcinogenic *N*-hydroxy-*N*-acetylaminoarenes by enzymatic deacetylation and transacetylation in liver. Biochim Biophys Acta 286:272–298, 1972

(*20*) BARTSCH H, DWORKIN C, MILLER EC, et al: Formation of electrophilic *N*-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-2-acetylaminobiphenyl. Biochim Biophys Acta 304:42–55, 1973

(*21*) KING CM: Mechanisms of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. Cancer Res 34:1503–1515, 1974

(*22*) KING CM, ALLABEN WT: Arylhydroxamic acid acyltransferase. *In* Enzymatic Basis of Detoxication (Jakoby WB, ed). New York: Academic Press, 1979, pp 187–197

(*23*) _____: The role of arylhydroxamic acid *N-O*-acyltransferase in the carcinogenicity of aromatic amines. *In* Conjugation Reactions in Drug Biotransformation (Aitio A, ed). Amsterdam: Elsevier/North Holland, 1978, pp 431–441

(*24*) WEEKS CE, ALLABEN WT, LOUIE SC, et al: Role of hydroxamic acid acyltransferase in the mutagenicity of *N*-hydroxy-*N*-2-fluorenylacetamide in *Salmonella typhimurium.* Cancer Res 38:613–618, 1978

(*25*) KING CM, ALLABEN WT, LAZEAR EC, et al: Influence of the acyl group on arylhydroxamic acid, *N-O*-acyltransferase-catalyzed mutagenicity, and metabolic activation of *N*-acyl-*N*-fluorenylhydroxylamines. *In* Biological Oxidation of Nitrogen (Gorrod JW, ed). Amsterdam: Elsevier, 1978, pp 335–340

(*26*) CARDONA RA, KING CM: Activation of the *O*-glucuronide of the carcinogen *N*-hydroxy-FAA by enzymatic deacetylation in vitro: Formation of FA–tRNA adducts. Biochem Pharmacol 25:1051–1056, 1976

(*27*) ALLABEN WT, BELAND FH: Acyltransferase-mediated binding of *N*-hydroxyarylamides to nucleic acids. Proc Am Assoc Cancer Res 20:67, 1979

(*28*) KING CM, SHAYMAN MA: Reaction in vivo of *N*-hydroxy-2-fluorenylacetamide with RNA and DNA of gastrointestinal tract and liver of the rat. Proc Am Assoc Cancer Res 15:42, 1974

(*29*) KING CM, OLIVE CW, CARDONA RA: Activation of carcinogenic arylhydroxamic acids by human tissues. J Natl Cancer Inst 55:285–287, 1975

(*30*) KING CM, OLIVE CW: Comparative effects of strain, species and sex on the acyltransferase- and sulfotransferase-catalyzed activations of *N*-hydroxy-*N*-2-fluorenylacetamide. Cancer Res 35:906–912, 1975

(*31*) KING CM, PHILLIPS B: *N*-Hydroxy-2-fluorenylacetamide: Reaction of the carcinogen with guanosine, ribonucleic acid, deoxyribonucleic acid, and protein following enzymatic deacetylation of esterification. J Biol Chem 244:6209–6216, 1969

(*32*) _____: Instability of fluorenylamide-substituted polynucleotides: Loss of carcinogen and production of an altered nucleic acid. Chem Biol Interact 2:267–271, 1970

(*33*) KING CM, SHAYMAN MA, THISSEN MR: Carcinogenic arylhydroxylamines: Reaction with phosphate of RNA and cleavage of the nucleic acid chain. Proc Am Assoc Cancer Res 16:475, 1975

(*34*) KRIEK E: On the mechanism of action of carcinogenic aromatic amines. I. Binding of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids in vivo. Chem Biol Interact 1:3–17, 1969

(*35*) IRVING CC, JANSS DH, RUSSELL LT: Lack of *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity in mammary gland and Zymbal's gland of the rat. Cancer Res 31:387–391, 1971

(*36*) KING CM, TRAUB NR, LORTZ ZM, et al: Metabolic activation of arylhydroxamic acid *N-O*-acyltransferase of rat mammary gland. Cancer Res 39:3369–3372, 1979

(*37*) KRIEK E: On the mechanism of action of carcinogenic aromatic amines. II. Binding of *N*-hydroxy-*N*-acetyl-4-aminobiphenyl to rat liver nucleic acids in vivo. Chem Biol Interact 3:19–28, 1971

(*38*) YAMAMOTO RS, WILLIAMS GM, RICHARDSON HL, et al: Effect of *p*-hydroxyacetanilide on liver cancer induction by *N*-hydroxy-*N*-2-fluorenylacetamide. Cancer Res 33: 454–457, 1973

(*39*) MALEJKA-GIGANTI D, GUTMANN HR, RYDELL RE: Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids. Cancer Res 33:2489–2497, 1973

(*40*) ALLABEN WT, WEEKS CE, TRESP NC, et al: Mammary tumor induction in the rat by *N*-acyl-*N*-2-fluorenylhydroxylamines: Structure-activity relationship. Fed Proc 37:2543, 1978

(*41*) IRVING CC: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26:1390–1396, 1966

(*42*) JARVINEN M, SANTTI RS, HOPSU-HAVU VK: Partial purification and characterization of two enzymes from guinea-pig liver microsomes that hydrolyze carcinogenic amides 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene. Biochem Pharmacol 20:2971–2982, 1971

(*43*) IRVING CC: Species and tissue variations in the metabolic activation of aromatic amines. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 211–227

(*44*) THORGEIRSSON SS, SCHUT HA, STAIANO N, et al: Mutagenicity of N-substituted aryl compounds in microbial systems. Natl Cancer Inst Monogr 58:229–236, 1981

Free Radicals in Arylamine Carcinogenesis[1]

Robert A. Floyd [2]

ABSTRACT—Free radical processes and their involvement in carcinogenesis is an unresolved question but one subjected to intense investigation recently. Using in vitro systems, we demonstrated that certain heme compounds combined with hydroperoxides catalyzed the oxidation of *N*-hydroxy-2-fluorenylacetamide (*N*-OH-2-FAA) to nitroxyl free radical intermediate which dismutated to form 2-nitrosofluorene (2-NF) and *N*-acetoxy-2-fluorenylacetamide (*N*-AcO-2-FAA). Ascorbate and certain antioxidants inhibited this reaction. Lipid hydroperoxides were effective substrates for this reaction, especially in target tissue, rat mammary gland parenchymal cells. Cytochrome P_{420} in the high-spin state catalyzed the reaction effectively but low-spin cytochrome P_{420} or P_{450} were ineffective. Recently, we found that 2-NF added covalently to unsaturated carbon–carbon double bonds of membrane lipids to form an adduct termed 2-nitrosofluorene lipid adduct (N-Ȯ-LAF), which, in its oxidized state, exists in the membrane as a nitroxyl free radical. This N-Ȯ-LAF undergoes reduction–oxidation changes in the natural membrane. Formation of N-Ȯ-LAF occurred in rat liver microsomal membranes by deacylation of *N*-OH-2-FAA, but the esterase inhibitor, paraoxon, prevented its formation from *N*-OH-2-FAA. The mutagenicity of 2-NF was enhanced in *Salmonella typhimurium* TA98 if the bacteria were cultured to contain more unsaturated membrane lipids. However, synthesized adducts were only slightly mutagenic.—Natl Cancer Inst Monogr 58: 123–131, 1981.

For some time, a few research workers have held that free radicals and cancer are associated. At best, this association was ill defined and regarded by most scientists as requiring much more investigation. The association between free radicals and neoplasms arose primarily as a result of the demonstrations that 1) X-rays produce cancer and 2) the primary action of X-rays involves free radicals.

Despite demonstrations, such as those provided by Nagata et al. (*1*), which implicate the importance of considering free radical processes in chemical carcinogenesis, few significant studies appeared until the early 1970's. The studies of Shamberger and his associates (*2–7*), Wattenberg (*8–11*), and Borchert and Wattenberg (*12*), though not involved with the chemistry of action of the chemical carcinogens, provided compelling evidence that the concept of free radicals and carcinogenesis must undergo careful scrutiny. Generally, Shamberger and Wattenberg demonstrated that the development of tumors initiated by a wide range of chemical carcinogens was inhibited by numerous synthetic and natural antioxidants. Even though antioxidants manifest broad spectra of biologic actions, the fact that they may be acting as free radical quenching agents must be kept in mind.

FREE RADICALS IN ARYLAMINE CARCINOGENESIS

At the time of the early antioxidant carcinogenesis work, Bartsch and Hecker (*13*) made the significant observation that when a system containing HRP, *N*-OH-2-FAA, and H_2O_2 was incubated, the more active carcinogens 2-NF and *N*-AcO-2-FAA were formed and that the nitroxyl free radical of *N*-OH-2-FAA (*N*-Ȯ-FAA) was present during the reaction. Text-figure 1 presents the scheme they postulated to explain the results: HRP is oxidized to compound 1 by H_2O_2 and is then reduced in two one-electron steps to the original oxidation state of the heme protein. These electrons are provided by *N*-OH-2-FAA which, upon oxidation, yield *N*-Ȯ-FAA, 2 molecules of which dismutate to yield 2-NF and *N*-AcO-2-FAA.

Further Examinations

A careful examination of the HRP–*N*-OH-2-FAA–H_2O_2 system established that the scheme presented by Bartsch et al. was correct. Thus we (*14*) demonstrated that 2 molecules of *N*-OH-2-FAA were converted to 1 molecule of 2-NF and 1 molecule of *N*-AcO-2-FAA. In addition, we showed that ascorbate inhibited the activation of *N*-OH-2-FAA in this system (*14*). The implications of these observations, i.e., activation of an intermediate carcinogen by a free radical intermediate route inhibited by an antioxidant, are important for one to understand the antioxidant action in carcinogenesis.

We extended our observations to models that were more meaningful biologically, and to this end, we studied the activation of *N*-OH-2-FAA using LAHP as the substrate and various heme compounds as catalysts (*15*). We found that the LAHP–methemoglobin or hematin–*N*-OH-2-FAA system activated *N*-OH-2-FAA into 2-NF and *N*-AcO-2-FAA stoichiometrically (text-fig. 2). Ascorbate also in-

Abbreviations: HRP = horse radish peroxidase; *N*-OH-2-FAA = *N*-hydroxy-2-fluorenylacetamide; H_2O_2 = hydrogen peroxide; 2-NF = 2-nitrosofluorene; *N*-AcO-2-FAA = *N*-acetoxy-2-FAA; LAHP = linoleic acid hydroperoxide; BHT = butylated hydroxytoluene; ESR = electron spin resonance; N-Ȯ-LAF= 2-nitrosofluorene lipid adduct; TME = 2,3-dimethyl-2-butene; $AgNo_3$ = silver nitrate; PCyFe = pentacyanoamine ferroate.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

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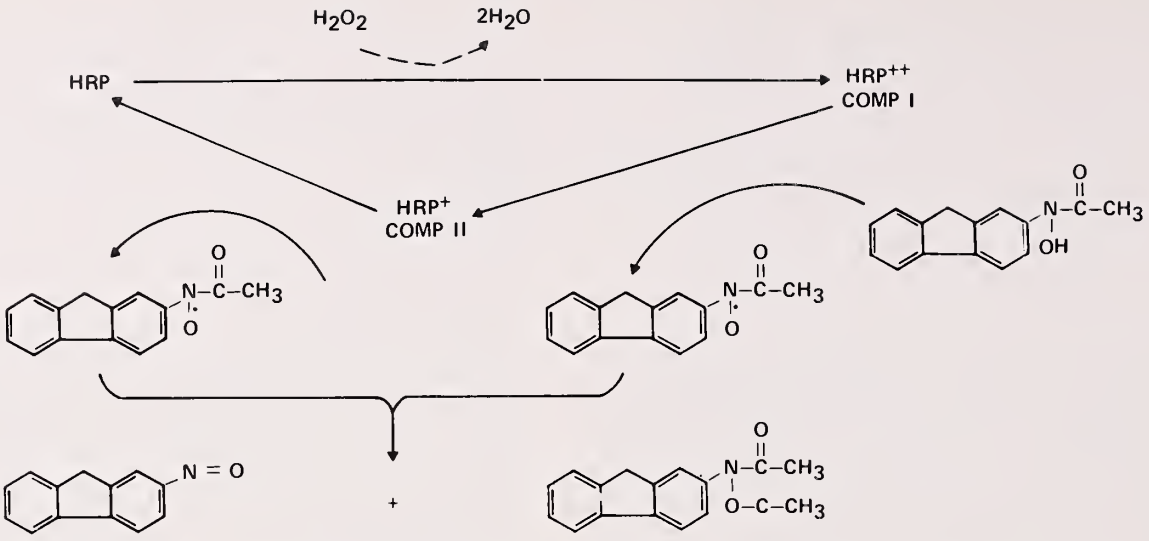
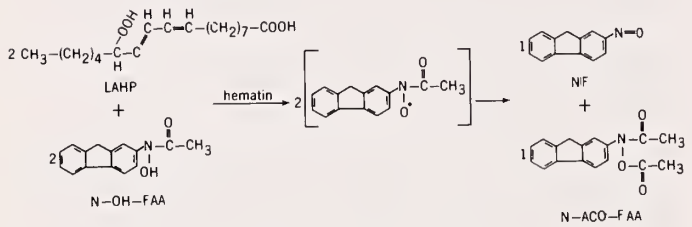
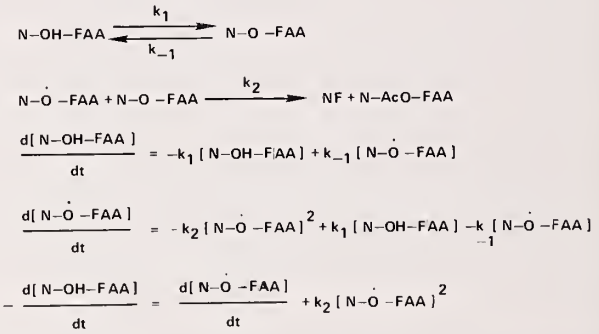


TEXT-FIGURE 1.—Scheme to explain HRP-catalyzed oxidation of *N*-OH-2-FAA to the nitroxyl free radical which yields after dismutation 2-NF and *N*-AcO-2-FAA. Comp I, Comp II = compound I and compound II of HRP, respectively.



TEXT-FIGURE 2.—Stoichiometry of the reaction showing hematin-catalyzed activation of *N*-OH-2-FAA. Text-figure is reproduced with permission from (*15*).



TEXT-FIGURE 3.—Rate equations explain the oxidative activation of *N*-OH-2-FAA through an obligatory nitroxyl free radical intermediate [redrawn from (*16*)].

hibited this reaction. A careful reexamination of the mechanism of the reaction established that indeed the nitroxyl free radical was an obligatory intermediate (*16*); the differential equations are presented in text-figure 3 (*16*). The dismutation constant of the nitroxyl free radical was $k_2 = 2.7 \times 10^5$ $M^{-1}sec^{-1}$. Thus under the particular conditions of the reaction investigated, the nitroxyl free radical of *N*-OH-2-FAA is a stable species, i.e., 0.1% of a 1 nM solution of this radical would be expected to dismutate in 1 second (*16*). Under physiologic conditions, agents such as ascorbate and reduced glutathione may reduce the radical back to *N*-OH-2-FAA and thus, in effect, may prevent the free radical route from occurring; this deduction remains to be proved.

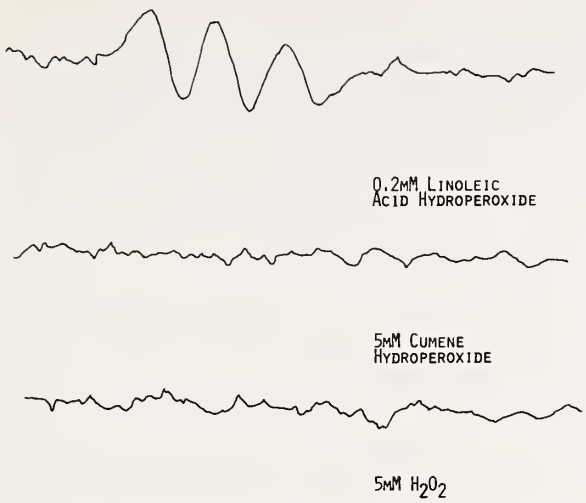
Biologic Relevance

In an attempt to make our results more meaningful, we tested whether *N*-OH-2-FAA is activated via the nitroxyl free radical route in parenchymal cells of rat mammary glands (*17*), a target tissue of the carcinogen. Using parenchymal cell homogenate, we found that: 1) The nitroxyl free radical of *N*-OH-2-FAA was present during the reaction, 2) 2-NF and *N*-AcO-2-FAA were formed, and 3) the reaction was inhibited by antioxidants such as propyl gallate, BHT, ascorbate, and reduced glutathione (*17*). We made other pertinent observations, the essence of which is summarized in text-figure 4. We noted that the high-spin form of P_{420} is an effective catalyst in the nitroxyl free radical route of *N*-OH-2-FAA activation and that as a substrate LAHP is much more effective than the organic peroxide cumene hydroperoxide or H_2O_2 (Reigh DL, Floyd RA: Unpublished observations). The importance of these findings and the relevance of the nitroxyl free radical activation route of *N*-OH-2-FAA are not clear.

NITROSOFLUORENE REACTIONS

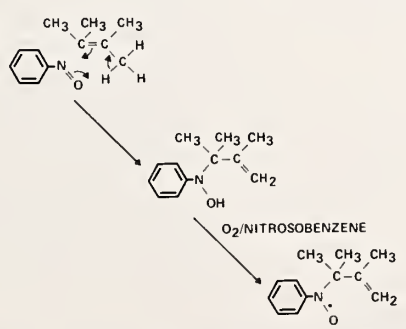
Unsaturated Lipid Components

Serendipitous observations have been valuable in advancing understanding of many areas, and the carcino-

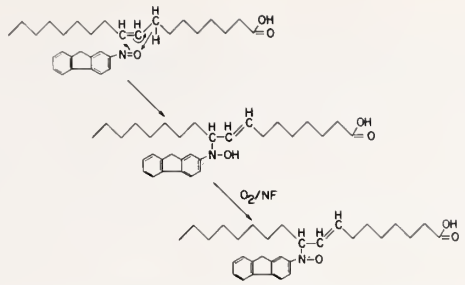


TEXT-FIGURE 4.—ESR spectra taken from rat liver P_{450}-enriched microsomes incubated with 0.14 mM *N*-OH-2-FAA to which had been added various hydroperoxides at the indicated concentrations. *N*-OH-2-FAA, 0.14 mM; P_{420}, 0.3 ml (high) prepared from P_{450} "particles"; 65 mM Tris-HCL, pH 7.4 (Reigh DL, Floyd RA: Unpublished observations).

genic process is no exception. Unexpectedly we (*18, 19*) made an observation that may be valuable in the eventual understanding of the nature of the processes involved in arylamine carcinogenesis: The evidence that 2-NF adds to membrane lipid components to form a covalent carcinogen-lipid adduct which, in the oxidized state, exists as a nitroxyl free radical compound termed N-Ȯ-LAF has been presented in (*18, 19*). The scheme to explain the data obtained was provided by Sullivan's observations (*20*) that demonstrated the addition of nitrosobenzene to TME (text-figure 5). Thus nitrosobenzene, in an "Alder-ene" type reaction, adds to TME to produce initially an N-hydroxy adduct which, either with oxygen or another nitrosobenzene, is oxidized to the nitroxyl free radical form. All the available data obtained can be explained by addition of 2-NF to membrane lipids in a manner equivalent to the nitrosobenzene-TME reaction and are presented schematically in the 2-NF-oleic acid reaction shown in text-figure 6.



TEXT-FIGURE 5.—Scheme proposed by Sullivan to explain the addition of nitrosobenzene to TME [redrawn from (*20*)].



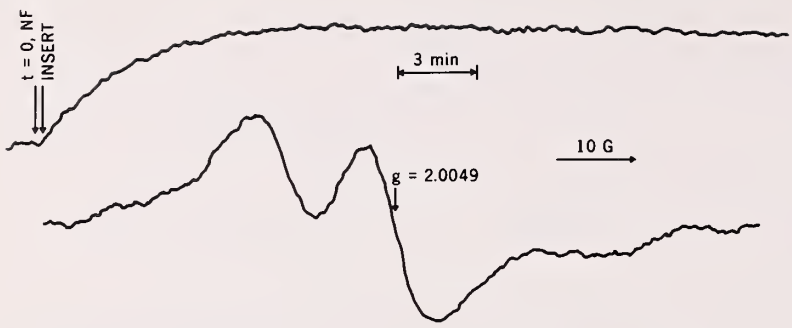
TEXT-FIGURE 6.—Scheme proposed to explain the addition of 2-NF to oleic acid. Text-figure is reproduced with permission from (*19*).

Natural Membranes

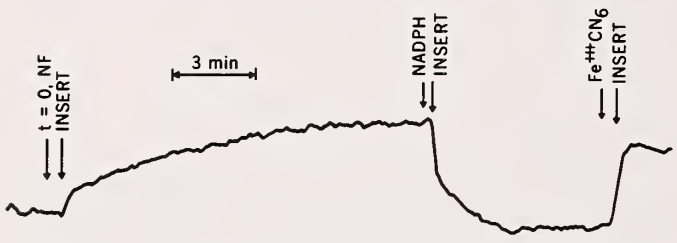
The 2-NF forms covalent adducts with lipid components of natural membranes (*19*). Text-figure 7 demonstrates the ESR spectrum, which is that of highly immobilized nitroxide, obtained when 2-NF interacts with rat liver microsomal membranes (*19*). The carcinogen-lipid adduct does not require the protein component to complete the reaction (*19*) because: 1) Liposomes formed from the total lipid extract of rat liver microsomes form the 2-NF-lipid adduct in approximately the same amount as that with intact rat liver microsomes per se; 2) heat-denatured microsomes were as effective as normal microsomes in forming the N-Ȯ-LAF. After more than 3 years of work in this area, we found that with every natural membrane (as well as artificial membranes containing unsaturated fatty acids) tested, formation of a nitroxyl free radical adduct occurs after reaction with 2-NF, so there is little doubt that if 2-NF is present, it would solubilize in the membrane because of its inherently more lipophilic nature, and then react with unsaturated fatty acid esters present in the membrane.

Nitrosofluorene-Lipid Adduct Redox State

When the 2-NF-lipid adduct changes reduction-oxidation states in the membrane, N-Ȯ-LAF is reduced to the hydroxylamine (N-OH-LAF) by the addition of NADPH to rat liver microsomes (text-fig. 8), but N-OH-LAF is oxidized back to N-Ȯ-LAF after addition of the oxidizing agent ferricyanide (*19*). The slight decrease in the amount of signal before and after reduction-oxidation is due to volume change. We discovered that other oxidizing agents, such as $AgNO_3$ (2-4 mM), are actually better than ferricyanide because in time ferricyanide, in contrast to $AgNO_3$, causes complete loss of the nitroxyl signal (Floyd RA: Unpublished observations). Also, the sulfhydryl binding agent *p*-hydroxymercuribenzoate retards the reduced pyridine nucleotide-mediated chemical reduction of N-Ȯ-LAF (*21*), but the nature of the molecular events involved is not known. This observation is similar to the demonstration that *p*-hydroxymercuribenzoate impedes the NADH-mediated reduction of nitroxyl spin probes in bacteria (*22*). However, the redox equilibrium of the cell is expected to be such that the N-Ȯ-LAF would exist predominately in the reduced state (N-OH-LAF). The presence of a perturb-



TEXT-FIGURE 7.—ESR spectrum (*lower trace*) of the adduct(s) formed when 2-NF reacts with rat liver microsomes. *Top trace* shows rate of formation of 2-NF lipid adducts. Magnetic field was set at top of first peak. Text-figure is reproduced with permission from (*19*).

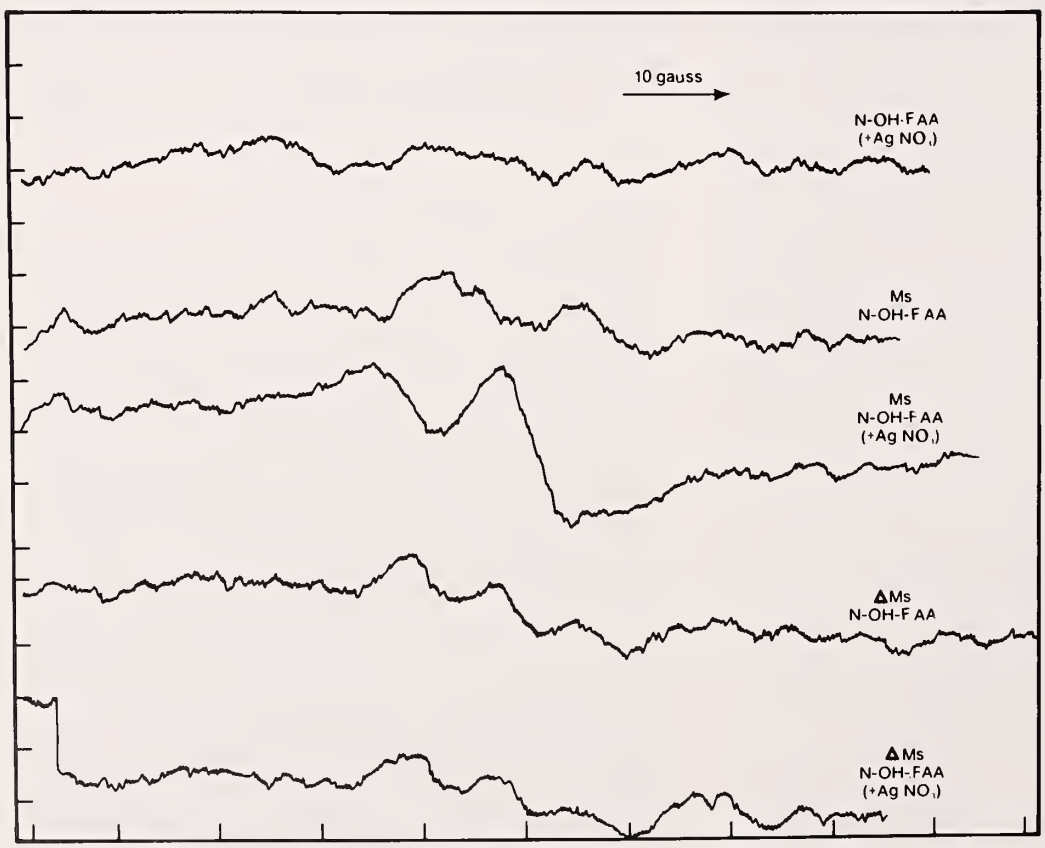


TEXT-FIGURE 8.—Time course of formation of 2-NF–lipid adduct(s) when carcinogen is added to rat liver microsomes; reduction of the carcinogen–lipid adduct after addition of reduced pyridine nucleotide and subsequent oxidation after addition of ferricyanide. Text-figure is reproduced with permission from (*19*).

ing agent in a natural membrane which can change redox states may have profound effects on the oxidation–reduction processes, such as electron transport, which naturally occur in a membrane. Also, if peroxidative processes occur in a natural membrane, then the presence of N-OH-LAF in that membrane would most likely influence the process, perhaps by delaying the overall peroxidative process. These aspects need more investigation.

DEACETYLATION OF N-HYDROXY-2-ACETYLAMINOFLUORENE

Does N-Ȯ-LAF form in vivo? This pressing question cannot be answered yet, but we (*23*) have come a step closer to answering it by demonstrating that N-Ȯ-LAF is formed from *N*-OH-2-FAA in rat liver microsomes (text-figure 9). The second trace shows the ESR signal obtained



TEXT-FIGURE 9.—ESR spectra show formation of 2-NF–lipid adduct formation when rat liver microsomes are incubated at 37° C in presence of *N*-OH-2-FAA. *Top trace* depicts the spectrum obtained when $AgNO_3$ is added to a buffer solution containing *N*-OH-2-FAA. *Next two traces* down show the signal obtained after incubating rat liver microsomes in the presence of carcinogen and after oxidation with $AgNO_3$. *Two lowest traces* were taken from heated (65° C for 15 min) rat liver microsomes treated as in the *two traces above* (Floyd RA: Unpublished observations).

after we incubated rat liver microsomes with *N*-OH-2-FAA. After oxidation with $AgNO_3$, third trace, a signal is evident that is equivalent to that obtained when 2-NF is added to rat liver microsomes (*23*). When *N*-OH-2-FAA was added to a buffer system in the presence of $AgNO_3$, it yielded no free radical signal (top trace). Heated microsomes could not catalyze N-Ȯ-LAF formation from *N*-OH-2-FAA (two bottom traces).

The powerful esterase inhibitor, *p*-nitrophenyl phosphate (paraoxon), prevented microsome-catalyzed N-Ȯ-LAF formation from *N*-OH-2-FAA (*23*), which is demonstrated by the results presented in text-figure 10. In the presence of paraoxon (top trace), little if any N-Ȯ-LAF was formed from *N*-OH-2-FAA. We also found that α-toluene sulfonate similarly inhibited microsome-catalyzed N-Ȯ-LAF formation from *N*-OH-2-FAA (*23*).

A rat liver microsomal enzyme catalyzes the deacetylation of *N*-OH-2-FAA to form *N*-OH-2-FA which then oxidizes to form 2-NF, and eventually N-Ȯ-LAF is formed. This reaction is illustrated clearly in text-figure 11. When TME is added to a solution containing rat liver microsomes and *N*-OH-2-FAA, a large signal from the 2-NF–TME adduct is noted. Oxidation with $AgNO_3$ emphasizes the large increase in signal when TME is present. However, heated microsomes were not active in the formation of 2-NF–TME adduct (two bottom traces).

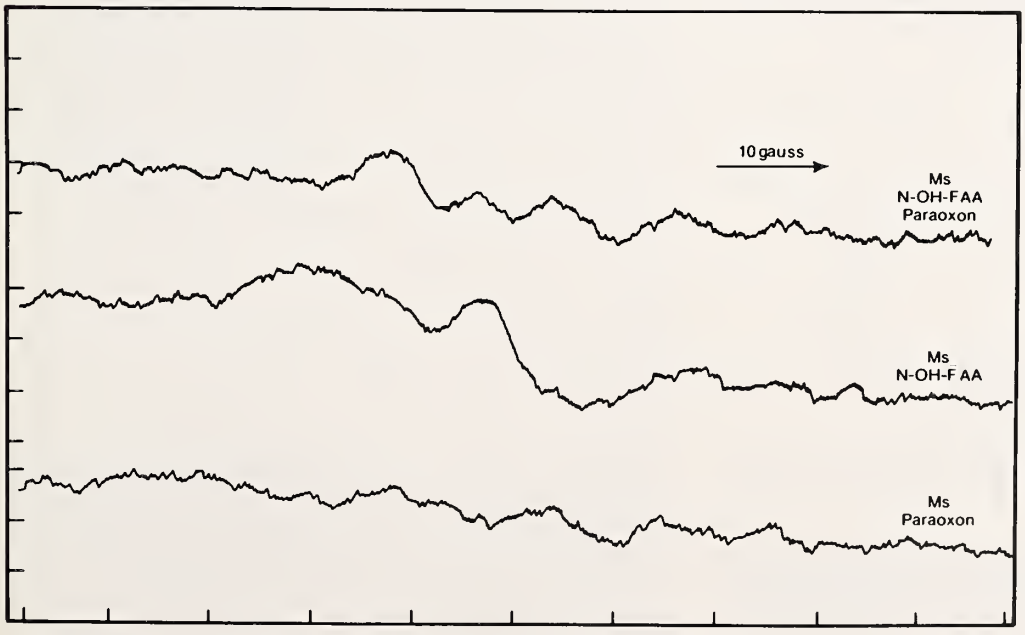
Because several steps are involved from deacetylation of *N*-OH-2-FAA to N-Ȯ-LAF formation, one could reasonably argue that the results of methods other than N-Ȯ-LAF measurement are necessary. Table 1 provides such corroborative data. Deacetylation of *N*-OH-2-FAA was measured by the absorbance of the purple complex formed from *N*-OH-2-FA combined with the ligand PCyFe (*24*). Using this alternate method of assessing *N*-OH-2-FAA deacetylation, our results corroborated those obtained with ESR methods, i.e., heating the microsomes and treating them with paraoxon completely abolished deacetylation of *N*-OH-2-FAA (*23*). *p*-Hydroxymercuribenzoate and BHT were only slightly inhibitory, whereas SKF-525A and cyanide were essentially without effect on the deacetylation. Our results, both those presented here and elsewhere (*23*), indicated that deacetylation of *N*-OH-2-FAA was not mediated by P_{450}.

Results of testing the hypothesis that PCyFe inhibits the formation of N-Ȯ-LAF are shown in text-figure 12. In the presence of PCyFe, an ESR signal indicated a hindered rotation nitroxyl free radical, but it was substantially different in spectral characteristics than was N-Ȯ-LAF (cf. bottom and second traces). We believe that the signal observed in the presence of PCyFe is the free radical of oxidized *N*-OH-2-FA liganded to PCyFe, which is perhaps attached by salt bridges to the microsomal surface.

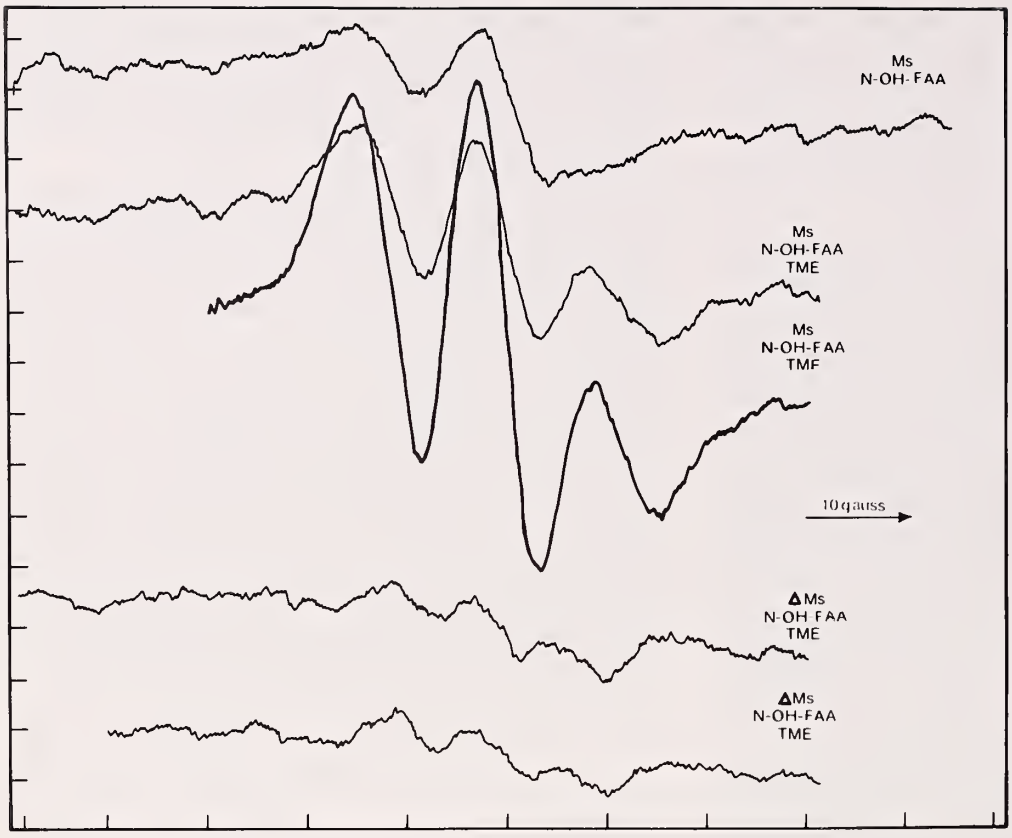
We tested several enzymes classified as esterases and determined that the carboxyl esterase from hog liver (type I) was active in deacetylating *N*-OH-2-FAA; the enzyme exhibited a K_m of 46 μM toward *N*-OH-2-FAA (*23*). Interestingly, the following enzymes were inactive in catalyzing the deacetylation: hog liver carboxyl esterase (type II), trypsin, chymotrypsin, ficin, papain, cathepsin D, *Naja naja* phospholipase, and acetylcholine esterase (*23*).

TABLE 1.—*PCyFe* N-*OH-FA*

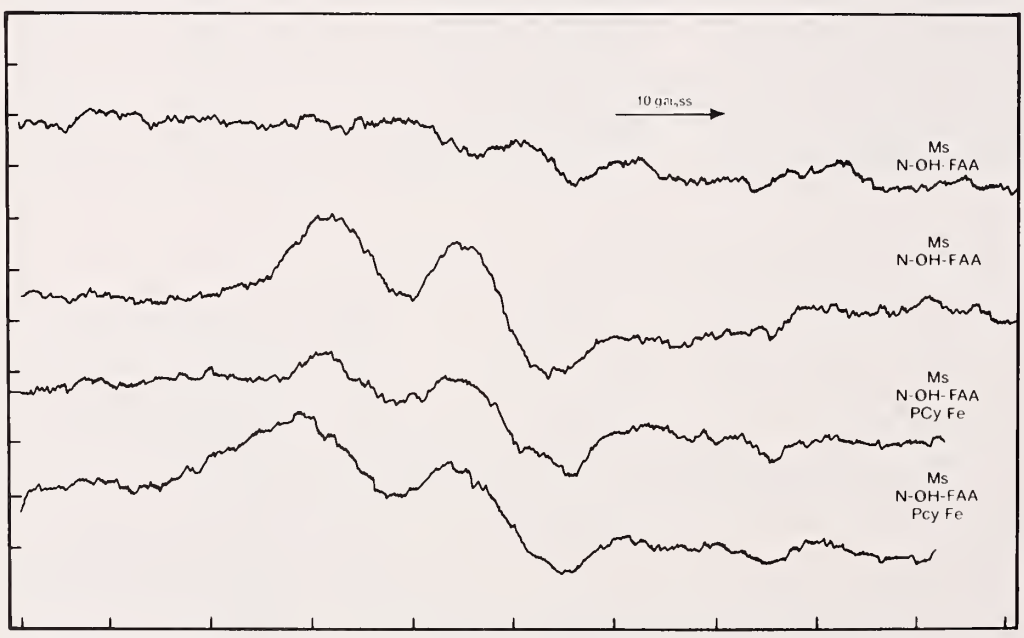
Treatment	Absorbance, λ = 575 nm	Percent control
Control	0.312	100.0
Control and ethanol	0.214	100.0
Heated microsomes	0.033	10.5
Paraoxon (0.22 mM)	0.046	14.7
p-Hydroxymercuribenzoate	0.220	70.5
BHT (0.66 mM)	0.163	76.2
SKF-525A (0.11 mM)	0.276	88.5
Sodium cyanide (2.2 mM)	0.263	84.3



TEXT-FIGURE 10.—ESR spectra demonstrate that paraoxon prevented rat liver microsome-catalyzed formation of 2-NF–lipid adduct(s) during incubation with *N*-OH-2-FAA. Microsomes were incubated in the presence of the carcinogen and then subsequently oxidized with $AgNO_3$ (text-fig. 9). Microsomes yielding the *top trace* had paraoxon in addition to the carcinogen. *Lowest trace* shows the spectrum obtained when the microsomes were incubated in the presence of paraoxon only (Floyd RA: Unpublished observations).



TEXT-FIGURE 11.—ESR spectra show that when rat liver microsomes are incubated in the presence of *N*-OH-2-FAA and TME, an enhanced signal formation is catalyzed by a heat-sensitive enzyme. *Top spectrum* was obtained after incubation of rat liver microsomes with the carcinogen and subsequent $AgNO_3$ oxidation. *Two next lowest spectra* were obtained as in the *top spectrum*, except TME only was present in the *second* and TME and $AgNO_3$ in the *third or middle spectrum*. Oxidation demonstrated the presence of an additional amount of the 2-NF-TME adduct. *Two lowest spectra* were taken as the two above (*second* and *third*) with the exception that heated microsomes were used (Floyd RA: Unpublished results).



TEXT-FIGURE 12.—ESR spectra demonstrate that incubation of rat liver microsomes in the presence of PCyFe and *N*-OH-2-FAA caused the appearance of a free radical substantially different than that present with the carcinogen only (Floyd RA: Unpublished results). In *second from top* and *bottom spectra*, $AgNO_3$ was also present.

MUTAGENESIS OF NITROSOFLUORENE-LIPID ADDUCTS

We began our attempt at answering the question concerning the relevance of N-O-LAF in arylamine carcinogenesis by assessing the mutagenicity of various 2-NF adducts (*25*). Table 2 demonstrates that the 2-NF-oleic acid adduct is mutagenic to the TA98 mutant of *Salmonella typhimurium* in the absence of the S-9 activating system. The 2-NF-TME adduct and the oleic acid adduct are equally potent (*25*), but each is only about 1 to 2% as potent a mutagen as 2-NF.

TABLE 2.—*Mutagenicity of the 2-NF adduct with methyl oleate toward* S. typhimurium *TA98*

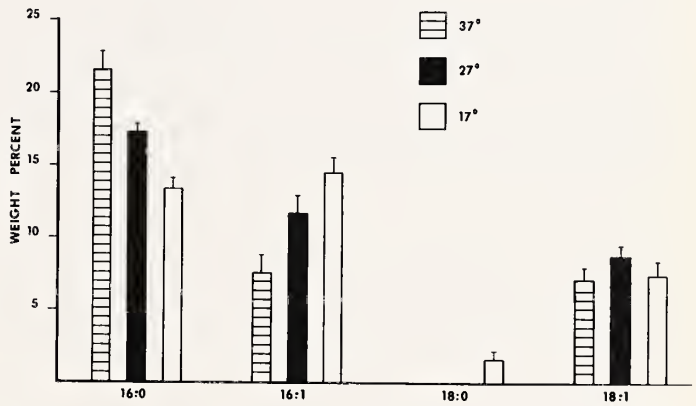
μg/Plate	Revertants/plate
100.00	1,493
50.00	1,088
20.00	555
10.00	127
2.00	47
0.20	20
0.02	17
None	24

In considering the mutagenicity of N-Ȯ-LAF in relation to the overall contribution of this adduct to arylamine carcinogenesis, one must keep in mind the results obtained by Bartsch et al. (*26*) with the myristate analogs of *N*-OH-2-FAA and *N*-AcO-2-FAA. They found that if the acetyl group of *N*-OH-2-FAA or the acetate ester of *N*-AcO-2-FAA was replaced by myristate, i.e., *N*-OH-MyFA and N-OMy-FAA, then the amount of sarcomas formed was enhanced dramatically, yet these myristate analogs were less mutagenic in the Ames' assay (*26*). Bartsch and his coworkers (*26*) explained the enhanced potency of the myristate analogs by their capacity to remain localized at the injection site for longer periods. Even though obvious molecular differences exist between the myristate analogs and N-Ȯ-LAF, lipophilicity is enhanced by the attachment of either myristate or a lipid molecule to 2-NF; this enhanced lipophilicity is most likely significant.

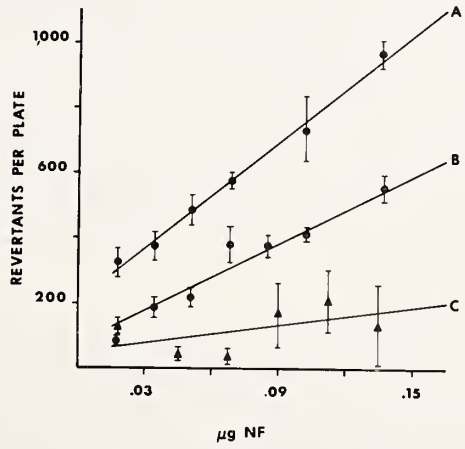
ROLE OF NITROSOFLUORENE-LIPID ADDUCT IN THE CARCINOGENIC PROCESS

We considered several ideas that we are currently testing to determine the importance of the N-Ȯ-LAF in carcinogenesis. First we thought that N-Ȯ-LAF (which would be expected to exist in the membrane) may effect homeostatic processes such as membrane potential, ion flow, and nutrient uptake. Then we considered the notion that N-Ȯ-LAF provides an effective means of juxtaposing a potential carcinogen with the inheritance macromolecules of the cell. Inasmuch as N-Ȯ-LAF would be in the membrane, movement by diffusion from the site of formation, presumably in the endoplasmic reticulum, would occur in the plane of the membrane to sites in the cell in apposition to membranes. Because nuclear membranes are thought to be contiguous with the endoplasmic reticulum, diffusion in the plane of the membrane, usually considered to be $D \simeq 10^{-8}$ cm^2/sec^{-1} (based on nitroxyl spin label studies), would within a second yield N-Ȯ-LAF molecules in the nuclear membrane. If N-Ȯ-LAF is present in the nuclear membrane, its interaction with chromatin is more likely, especially if one believes that chromatin-nuclear membrane interactions do occur in vivo.

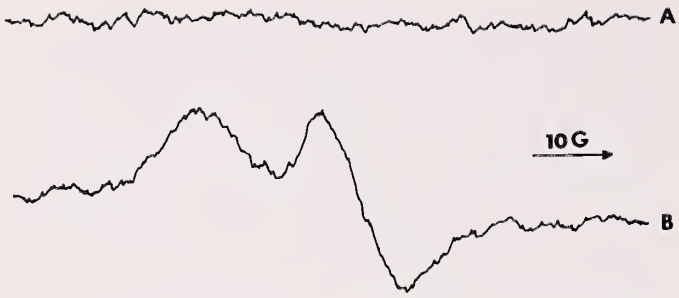
We wrestled with the notion that the inheritance macromolecules interact with membranes containing carcinogens either as covalently bound lipid products or as free carcinogens and attempted to test the importance of the ideas by using the Ames' tester strains of bacteria (*25*). A bacteria model has an advantage because the DNA replication site is at the membrane interface. Generally, when bacteria are grown at lower temperatures, they have an elevated unsaturated lipid content, which is also true with *S. typhimurium* TA98 (*21*). That 16:1 fatty acids increased at the expense of the 16:0 fatty acids when the bacteria were grown at lower temperatures (*21*) is illustrated in text-figure 13. Membrane microviscosity decreased as the unsaturated lipids increased as would be expected. The dose-response curve, i.e., the number of mutations versus the amount of 2-NF added per petri plate, is given in text-figure 14. More mutations occurred for the bacteria grown at 27° C than at 37° C, but few were observed at 17° C. The anomaly of the bacteria grown at 17° C was attributed to penetration problems with these organisms, i.e., a lack of permeases (*21*). Bacteria which have been incubated with 2-NF in the presence of *p*-hydroxymercuribenzoate to slow down reduction of N-Ȯ-LAF yield an ESR spectrum indicative of the N-Ȯ-LAF (*21*), as shown in text-figure 15. The uptake of 2-NF, as measured by [^{14}C]2-NF and the amount of N-Ȯ-LAF formed, correlated with the number of mutations observed (table 3). All the data presented on bacterial systems neither prove nor disprove the correctness of the



TEXT-FIGURE 13.—Fatty acid composition of *S. typhimurium* TA98 grown at 37°, 27°, and 17° C (*21*).



TEXT-FIGURE 14.—Revertant colonies formed of *S. typhimurium* TA98 as a function of 2-NF added per plate for bacteria grown at 27° (A), 37° (B), and 17° (C). Text-figure is reproduced with permission from (*21*).



TEXT-FIGURE 15.—ESR spectra obtained before (A) and after (B) 2-NF was added to a bacterial suspension of *S. typhimurium* TA98. G = gauss. Text-figure is reproduced with permission from (*21*).

TABLE 3.—*Binding of 2-NF to* S. typhimurium *TA98 grown at different temperatures*

2-NF/10^6 cells	Growth temperature		
	37° C	27° C	17° C
nM/10^6 cells[a]	6.3	91.0	0.1
dpm/10^6 cells[b]	48.1	192.0	5.2

[a] Concentrations were determined by comparison of ESR spectra of the 2-NF–lipid free radical adducts to a spectrum of a known concentration of the free radical standard 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

[b] Radioactivity measurements were done on bacteria incubated with [^{14}C]2-NF (sp act, 2.5 μCi/mg).

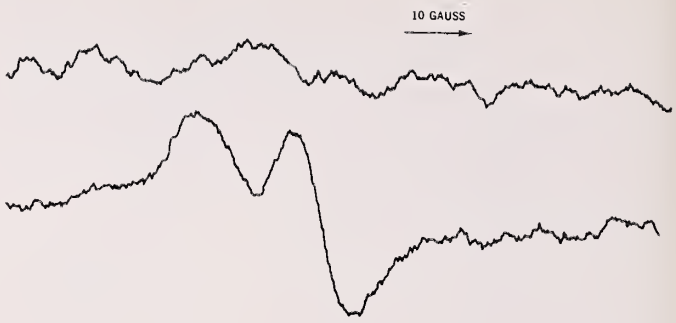
N-Ȯ-LAF membrane-inheritance macromolecule concept. In fact, these results are only a start in its investigation and are only background information necessary to begin testing the concept. However, they do indicate the direction in which our research is proceeding.

In addition to the two possibilities mentioned above that N-Ȯ-LAF has on arylamine carcinogenesis, there are two other considerations: 1) N-Ȯ-LAF may act as a storage form for 2-NF (we conceive that 2-NF would be formed from a retro Alder-ene reaction). 2) It may dismutate in the membrane like the nitroxyl of *N*-OH-2-FAA does to produce 2-NF and the lipid ester of N-Ȯ-LAF. Also, N-Ȯ-LAF may act in some unknown manner as a promoter in arylamine carcinogenesis.

Other arylamine carcinogens form a c-nitroso lipid adduct. Text-figure 16 shows that incubation of rat liver microsomes with *N*-hydroxy-4-acetylaminobiphenyl yields an ESR signal indicative of the c-nitroso-lipid adduct.

CONCLUSIONS

From this discourse on free radical events and arylamine carcinogenesis (most of which by necessity has been a review of my work), the question of the involvement of free radical events in carcinogenesis can be answered with a hesitant "maybe!" Based on work we conducted, the intermediate arylamine carcinogen would appear to be activated, under appropriate conditions, from *N*-OH-2-FAA to *N*-AcO-2-FAA and 2-NF by a nitroxyl free radical



TEXT-FIGURE 16.—ESR spectra demonstrate the presence of the 4-nitrosobiphenyl–lipid adduct(s) present after incubation of rat liver microsomes with *N*-hydroxy-4-acetylaminobiphenyl. *Top* and *lower spectra* are before and after, respectively, the addition of ferricyanide as an oxidizing agent (Steward JE, Floyd RA: Unpublished results).

intermediate. The reaction is sensitive to antioxidants, but whether it actually occurs in vivo has not been proved. However, our previous work indicated that deacetylation of *N*-OH-2-FAA occurs in vivo and eventually leads to an N-Ȯ-LAF, the role of which in carcinogenesis is not known. Several possible effects that this lipid adduct may have are that it may: 1) influence homeostatic properties controlled by membranes, 2) be a form to facilitate the juxtaposing carcinogen with inheritance macromolecules, 3) act as a storage for 2-NF, and 4) act as a promoter, co-carcinogen, or accelerator to the carcinogenic process. Much work remains for adequate assessment of the importance of this adduct in arylamine carcinogenesis.

REFERENCES

(*1*) NAGATA C, KODAMA M, IOKI Y, et al: Free radicals produced from chemical carcinogens and their significance in carcinogenesis. *In* Free Radicals and Cancer (Floyd RA, ed). New York; Marcel Dekker. In press

(*2*) SHAMBERGER RJ, RUDOLPH G: Protection against cocarcinogenesis by antioxidants. Experientia 22:116, 1966

(*3*) SHAMBERGER RJ: Relationship of selenium to cancer. I. Inhibitory effect of selenium on carcinogenesis. J Natl Cancer Inst 44:931–936, 1970

(*4*) _____: Increase of peroxidation in carcinogenesis. J Natl Cancer Inst 48:1491–1497, 1972

(*5*) SHAMBERGER RJ, BAUGHMAN FF, KALCHERT SL, et al: Carcinogen-induced chromosomal breakage decreased by antioxidants. Proc Natl Acad Sci USA 70:1461–1463, 1973

(*6*) SHAMBERGER RJ, RUKOVENA E, LONGFIELD AK, et al: Antioxidants and cancer. I. Selenium in the blood of normals and cancer patients. J Natl Cancer Inst 50: 863–870, 1973

(*7*) SHAMBERGER RJ, ANDREONE TL, WILLIS CE: Antioxidants and cancer. IV. Initiating activity of malonaldehyde as a carcinogen. J Natl Cancer Inst 53:1771–1773, 1974

(*8*) WATTENBERG LW: Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons of phenolic antioxidants and ethoxyquin. J Natl Cancer Inst 48:1425–1430, 1972

(*9*) _____: Inhibition of chemical carcinogen-induced pul-

monary neoplasia by butylated hydroxyanisole. J Natl Cancer Inst 50:1541–1544, 1973

(*10*) _____: Inhibition of chemical carcinogenesis by butylated hydroxanisole (BHA) and thiuram disulfide derivatives. Proc Am Assoc Cancer Res 14:7, 1973

(*11*) _____: Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by several sulfur-containing compounds. J Natl Cancer Inst 52:1583–1587, 1974

(*12*) Borchert P, Wattenberg LW: Inhibition of macromolecular binding of benzo[*a*]pyrene and inhibition of neoplasia by disulfiram in the mouse forestomach. J Natl Cancer Inst 57:173–179, 1976

(*13*) Bartsch H, Hecker E: On the metabolic activation of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene. III. Oxidation with horseradish peroxidase to yield 2-nitrosofluorene and *N*-acetylaminofluorene. Biochim Biophys Acta 237:567–578, 1971

(*14*) Floyd RA, Soong LM, Culver PL: Horseradish peroxidase/hydrogen peroxide-catalyzed oxidation of the carcinogen *N*-hydroxy-*N*-acetyl-2-aminofluorene as effected by cyanide and ascorbate. Cancer Res 36:1510–1519, 1976

(*15*) Floyd RA, Soong LM, Walker RN, et al: Lipid hydroperoxide activation of *N*-hydroxy-*N*-acetylaminofluorene via a free radical route. Cancer Res 36:2761–2767 1976

(*16*) Floyd RA, Soong LM: Obligatory free radical intermediate in the oxidative activation of the carcinogen *N*-hydroxy-2-acetylaminofluorene. Biochim Biophys Acta 498:244–249, 1977

(*17*) Reigh DL, Stuart M, Floyd RA: Activation of the carcinogen *N*-hydroxy-2-acetylaminofluorene by rat mammary peroxidase. Experientia 34:107–108, 1978

(*18*) Floyd RA: Free radicals produced in a nitrosofluorene-unsaturated lipid reaction. Experientia 33:197–198, 1977

(*19*) Floyd RA, Soong LM, Stuart MA, et al: Free radicals and carcinogenesis. Some properties of the nitroxyl free radicals produced by covalent binding of 2-nitrosofluorene to unsaturated lipids of membranes. Arch Biochem Biophys 185:450–457, 1978

(*20*) Sullivan AB: Electron spin resonance studies of a stable arylnitroso–olefin adduct free radical. J Org Chem 31: 3811–3817, 1966

(*21*) Hampton MJ, Floyd RA, Clark JB, et al: Binding and mutagenicity studies with 2-nitrosofluorene on *Salmonella typhimurium* TA98 grown at different temperatures. Mutat Res 69:231–239, 1980

(*22*) Baldassare JJ, Robertson DE, McAfee AG, et al: A spin-label study of energy-coupled active transport in *Escherichia coli,* membrane vesicles. Biochemistery 13: 5210–5214, 1974

(*23*) Steward JE, Floyd RA: The deacetylation of *N*-hydroxy-2-acetylaminofluorene by rat liver microsomes and carboxyl esterase. Cancer Biochem Biophys 5:47–53, 1980

(*24*) Schut HA, Wirth PJ, Thorgeirsson SS: Mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system: The role of deacetylation by liver and kidney fractions from mouse and rat. Molec Pharmacol 14:682–692, 1978

(*25*) Sridhar R, Hampton MJ, Steward JE, et al: Studies on the mutagenicity and electron spin resonance spectra of nitrosofluorene–lipid adducts. Appl Spectroscopy 34: 289–293, 1980

(*26*) Bartsch H, Maloveille C, Stich HF, et al: Comparative electrophilicity, mutagenicity, DNA repair inductive activity and carcinogenicity of some N- and O-acyl derivatives of *N*-hydroxy-2-aminofluorene. Cancer Res 37:1461–1467, 1977

Discussion III [1]

J. R. Gillette: The session is now open for questions.

G. Williams: I wanted to comment on the correlation that Dr. E. Weisburger reported between the ability of various species to excrete 2-FAA by either the feces or the urine and the carcinogenicity. For example, she noted that over a 2-day period, the rat would excrete 24% of the administered dose in the feces, whereas the hamster excreted only 3.5%. Conversely in the urine, the rat excreted 48–65% of the dose, whereas the hamster excreted 90%. This kind of metabolic study would lead one to predict that hamsters would be more susceptible to bladder carcinogenesis by aromatic amines and indeed that appears to be true. At our Institute, we conducted several studies on the carcinogenicity of DMAB in both the hamster and, in a study by Dr. Fiala, the rat. In the hamster, it produces primarily bladder tumors, a finding that correlates with the greater urinary excretion of aromatic amines, but in the rat, DMAB produces primarily colon cancers. I think that this kind of a study exemplifies to me the kind of correlation that we want to make between metabolic parameters and carcinogenicity. On the other hand, I am puzzled by the fact that it is frequently alleged that there would be a correlation between the *N*-acetyltransferase activity and bladder carcinogenesis because the hamster has the greatest activity of *N*-acetyltransferase among various species that have been studied. Yet the hypothesis is that *slow* acetylators should be more susceptible to bladder carcinogenesis. I would like to know how the people who reported on the acetyltransferase activity reconcile this fact. I would also like to make a further comment that really goes back a bit to something that Dr. Gutmann had described: the competition between the 3-FAA and 2-FAA binding in metabolism by microsomes. Dr. Elizabeth Miller raised a question at that time as to what would be the effect of administering these two compounds simultaneously. The implication was left that carcinogenesis would be inhibited; I would like to point out that that is frequently not so. Particularly, for example, in the studies we have done with DMAB (Dr. Fiala's study) when the metabolic inhibitor disulfiram is administered concurrently with it, all that happens is that the organ site is shifted from the colon to the urinary tract. This is a principle that I think was first clearly demonstrated in Dr. Magee's laboratory by Peter Swann, who claimed to show that in animals on a protein-deficient diet, although the liver metabolism of dimethylnitrosamine was greatly diminished, the organotropism was simply shifted to the kidney and essentially a 100% incidence of kidney tumors resulted. In these studies on metabolic inhibition of activation of carcinogens, the net effect may really only be a shift in the organ site rather than a true inhibition of carcinogenicity.

M. Poirier: I would like to know if the nature or the quantity of the lipids added to the in vitro-activated system altered the ratio of C-hydroxylation to N-hydroxylation.

P. Lotlikar: In the data I showed, the C-hydroxylation was not completely reconstituted. If you add lipids, the microsomes had no more C- than N-hydroxylation.

S. S. Thorgeirsson: I want to emphasize the point that we do in fact have multiple forms of cytochrome P_{450}, and that these forms are site specific with respect to hydroxylation on the substrate. We have examined this question of site specificity of different P_{450} forms with respect to aromatic amines. These studies were done in collaboration with Dr. Eric Johnson. We examined C- and N-hydroxylation of 2-FAA with 4 highly purified P_{450} forms isolated from rabbit liver. Of these 4 forms, only 1, form 4, catalyzed N-hydroxylation. The others, i.e., forms 3 and 6, catalyzed solely the C-hydroxylation at the 7-position on the fluorene ring. Form 2, the form that is induced by phenobarbital in the rabbit, did not hydroxylate 2-FAA at all. Here we have an example of what appears to be a single cytochrome catalyzing the first and the obligatory step in the metabolic activation of 2-FAA and perhaps also for other aromatic amines. Other cytochromes, in all likelihood under different genetic regulation, are responsible for the detoxification, that is to say, the C-hydroxylation.

J. Weisburger: Let us take a 15-second commercial to remind this young audience that in 1958 Dr. Elizabeth Weisburger and I published a short paper in *Science* on the basis of the dramatic difference in the then known metabolites of 2-FAA in the guinea pig versus the rat. Way back in 1958, we said that there must be multiple forms of hydroxylating enzymes.

Gillette: We said it in 1956.

J. Scribner: In one of your slides, Dr. Irving, you showed that the slope for the reactivity of the glucuronide of *N*-OH-2-FAA was steeper than the slope at approximately the same pH of the other 3 groups of glucuronides. I would like to suggest that what is happening is that you are actually getting a change in the reaction characteristics of the species as you switch from the reaction with the acetyl aryl nitrenium to the pure aryl nitrenium ion. With the other 3 compounds, the primary reactivity in both the acetylate and nonacetylated compound is primarily that of nitrogen. However, with the stilbene, the amide activity is probably concentrated for all the nucleophiles at the double bond region, but in the amine, I think you will find, and this is educated speculation, that their reactivity will shift toward the nitrogen end.

Abbreviations: *N*-2-FAA = *N*-2-fluorenylacetamide; DMAB = 2′, 3-dimethyl-4-aminobiphenyl; OH = hydroxy; 2-FA = 2-fluorenamine.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

C. Irving: All I can say is that this is possible. We did not investigate it any further than I reported.

Gillette: Dr. Hinson's paper is open for discussion.

J. Weisburger: Did you do a control experiment using ascorbate without adding *N*-OH-2-FAA? The reason I say this is because you find a dichotomy between mutation which increased and the adduct formation which decreased. Dr. Hans Stich, in 1975 and even more recently, detected that ascorbate in the presence of certain metal ions was mutagenic and that mutagenicity could account for all your findings if you use that as a control.

J. Hinson: We did the controls. There was no mutagenesis in the presence of ascorbate in the absence of *N*-OH-2-FAA.

R. Floyd: I have another question concerning the control. Did you add catalase to any of the systems in which you specifically had ascorbate and the various cytosol fractions?

Hinson: We did not add catalase.

Gillette: I might point out there is a lot of catalase in the bacteria. I doubt that would be a point here.

M. W. Lieberman: At another recent symposium in a discussion on mechanisms of ascorbate reaction, Dr. Arthur Markell of Texas A & M pointed out that ascorbate can add to the presence of metals as an oxidizing agent and not as a reducing agent. I wondered if there might not be a small amount of an oxidizing product formed with the presence of ascorbate in the system?

Hinson: We quantitated all the metabolites.

Gillette: That is not what he is talking about. He is referring to the Fenton reaction, and we have studied that; actually, it decreases mutagenicity because it is so cytotoxic.

C. King: One of the questions I have for you, Dr. Hinson, is related to technical approaches, i.e., the use of protein rather than nucleic acid for adduct formation. It is really ill advised when you can have the arylamine moiety without the attached acetyl reacting. Use of both ring and acetyl labels simultaneously in the same experiment has been most useful. Therefore, you do not have to rely on acetate. I think the use of nucleic acid as a trapping agent is much to be preferred so as to avoid reactions of hydroxyamines and nitroso compounds in those situations. We may perhaps help to clarify it, inasmuch as the use of acyltransferase as we have discussed before is subject to inactivation because of its sulfhydryl problems. Any metal ions you have in your supernatant are "complexed out," which will drastically affect the level of this enzyme.

Hinson: We used the formation of acetic acid, under our experimental conditions, to estimate the binding of the arylamine moiety.

King: I would agree that measuring acetic acid is one way of doing this. I am simply saying that by using polynucleotides as your trapping agents, you have a much better controlled and more sensitive way of doing this instead of using binding to proteins.

Dr. Gillette: We will now discuss Dr. King's paper.

King: I can respond to Dr. Williams' question about the *N*-acetyltransferase in the hamster. Although we have not investigated the phenotypic relationship between the acetyl-CoA enzyme and the *N-O*-acyltransferase in any species other than the rabbit, it is possible that similar relationships exist in other species. On the other hand, I think we have to realize that probably a number of mechanisms we already know can be responsible for tumor induction by these compounds. For example, the sulfate in liver is obviously important, but in whatever way, that may still be open to debate. Also, the acyltransferase may be important in the mammary gland. Certainly neither seem to be important in the bladder of the dog. I think we should not be monolithic in our concepts, and the other point worth repeating is the possibility that the slow acetylators may not be at greater risk, simply because we do have, at least in the liver, a greater metabolic activation simultaneous with this higher acetylation. The problem is we are not even sure at this point, and I am being frank about this, that activation extends to other tissues and to this phenotypic relationship. The reactions from aromatic amines (certainly in terms of metabolic activation) dictate that the metabolic activation occurs in the target tissue. Much is to be done in this area. There is the possibility that in certain animals a great deal of metabolic activation takes place in high acetylator types. I might also repeat that in the gastrointestinal tract of the rat, acyltransferase is in the colon as well as the small intestine. If we study this more definitively, e.g., the distribution of the proper substrates, the presence of one or more metabolic activation systems, the concept of repair with tissue replication, and DNA synthesis we should not expect to find any simple direct correlation.

W. W. Weber: I would like to respond to Dr. Williams' question particularly in relation to Dr. Lotlikar's results. Dr. Lotlikar found that in Danish smokers, there was a slight excess of slow acetylators among the cancer patients, whereas in another similarly constituted population from Sweden, he found no significant excess. Also, in his study of a Wisconsin population, he found no differences. Now I want to amplify something that has not been brought out here before. First, the differences in the control values in the population in Denmark were not large, and second, racial and geographic variations in the incidence of rapid and slow acetylators around the world are important. It is most difficult to discern the significance of the small differences between a disease population and a so-called national control population. For instance, about 90 or 95% of the Japanese are rapid acetylators, but in certain parts of the Middle East, e.g., in Egypt and some tribes in Israel, the opposite is true. This problem constantly recurs in the study of populations when we deal with small differences between the sick and the control populations. In reference to the data on rabbits, because the acyltransferase and the *N*-acetyltransferase activities are correlated and both are high, one might ask why the rabbit is not more susceptible. I would agree with the qualification that Dr. King made that not all mechanisms are the same in different tissues for the formation of cancers.

Gillette: Does anyone have any questions for Dr. Floyd?

Lotlikar: Did the P_{420} act like hematin in your experiments?

Floyd: Quite right. It may be just catalytic; I cannot be certain now.

D. Malejka-Giganti: Dr. Floyd, I had the impression from some of your abstracts published in the past that you

or your group purified to some extent the peroxidase preparation of the rat mammary gland. It was like an ammonium precipitate step and it seemed to refer to the whole homogenate. I would like to find out whether this homogenate was from normal or lactating rats, and I would appreciate more detail on the peroxidase preparation. The second question is: Did you find your membrane-bound radical with 2-nitrosofluorene with mammary gland microsomes?

Floyd: In answer to your question about the mammary gland preparation, we prepared the cells first and then homogenized them. We have not performed the study on the reaction of the radical adduct to the lipid in the microsomes from the mammary gland. It would be a nice experiment to do, and we will try to do it in the future.

Cohen: I was interested in your data on the interaction of nitrosofluorene with the microsomal membranes, especially the lipid fraction. You also mentioned the possibility of this mechanism being important in promotion. Do you have any evidence that there is an interaction with the cell membrane rather than with just microsomal membranes because evidence is accumulating that promotion may be acting through a membrane interaction?

Floyd: The plasma membrane from the rat liver is what you are talking about. We have not isolated that per se but every membrane system that we have studied, including chylomicrons from humans and an endless array from bacteria, *Salmonella,* for instance, form the adduct. For example, there is a considerable amount of reductase that will keep the preparation in the reduced state. If you are willing to work with it and delay the sulfhydryl dehydrogenase, you can see the signal on the ESR machine. In every membrane system we have added 2-nitrosofluorene you can see the signal so it would be incredibly surprising if we did not see it. If indeed you see *N*-OH-2-FAA circulating in the blood, and if you have that deacetylated at the plasma membrane level, then I see no reason why the binding should not occur there. If the membrane components are recirculated throughout the cells, such as in a phospholipid exchange protein, the actual diffusion would be at 10^{-8}. I fail to see why it would not go to any part of that cell. There is just an immense amount of work here to do, and I might add that when you try to isolate, let us say you react your membranes with 2-nitrosofluorene, then why in the world can you not determine the reacting components? To clarify and not lead anyone astray, only a small amount of the membranes have reacted, but, definitely, in my opinion, it has to react if the 2-nitrosofluorene is present. I would be most surprised if it did not.

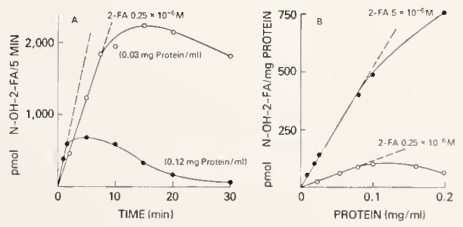
Gillette: Drs. C. Razzouk and M. Roberfroid of the Catholic University of Louvain in Belgium,[2] will now discuss guinea pig resistance to carcinogenicity by 2-FAA.

M. Roberfroid: The guinea pig is known to be resistant to the carcinogenic effect of both 2-FAA (*1, 2*) and 2-FA (*3*). The resistance to the effect of 2-FAA has been attributed to the inability of this animal species to N-hydroxylate the arylamide (*4*), but no explanation has been given to the resistance toward 2-FA. However, a few reports have demonstrated that the guinea pig liver microsomes have a low but detectable *N*-hydroxylase activity which is inducible by 3-methylcholanthrene pretreatment in vivo and which is increased when paraoxon, an inhibitor of microsomal diacetylase, is added in vitro to the incubation medium (*5, 6*). We analyzed the microsomal-mediated metabolism of 2-FAA and 2-FA by applying the specific and highly sensitive assay using guinea pig liver that we recently developed (*7, 8*) for measuring the hydroxy derivatives of these compounds. We herewith present our main conclusions of this study (*9*).

We did all the experiments reported here by incubating microsomes prepared by subcellular fractionation of liver from male 400- to 450-g guinea pigs, obtained from the Proefdierencentrum of the Catholic University of Louvain, which were fed ordinary laboratory chow up to 24 hours before being killed.

The conditions of incubation and details of the gas chromatographic assay are reported elsewhere (*10*). As shown in text-figure 1, guinea pig liver microsomes efficiently catalyzed the N-hydroxylation reaction of 2-FA used at a concentration of 0.25 10^{-6} M. Both the time of incubation and the concentration of proteins in the assay influence the *N*-hydroxylase activity. One may conclude from such experiments that if guinea pig liver microsomes N-hydroxylate 2-FA, they are also able to metabolize it rapidly. In relation to the rat, hamster, and mouse (*10*), the guinea pig is the most active species in N-hydroxylating arylamine (*9*). With 2-FAA as a substrate, guinea pig liver microsomes catalyzed the formation of only trace amounts of *N*-OH-2-FAA (~0.5 pmol/min/mg protein). This activity is, however, largely increased by the in vitro addition of paraoxon (5.10^{-5} M) plus 10^{-1} M sodium fluoride, which inhibit the microsomal diacetylase. In the presence of these two enzyme effectors, guinea pig liver microsomes N-hydroxylate 2-FAA as a function of protein concentration and incubation period (text-fig. 2). In terms of its maximum volume, its microsomal enzyme activity is almost equal to that of the rat liver.

Again, however, the *N*-OH-metabolite disappears rapidly from the incubation medium when protein concentration is increased or incubation is prolonged. Like *N*-OH-2-FA, *N*-OH-2-FAA appears to be metabolized further by guinea pig liver microsomes even in the presence of deacetylase inhibitors. This capacity of guinea pig liver microsomes to metabolize the N-OH-compounds further is clearly demonstrated by the experiments reported in text-



TEXT-FIGURE 1.—Microsomal 2-FA *N*-hydroxylase activity of guinea pig liver as a function of time of incubation (A) and protein concentration (B).

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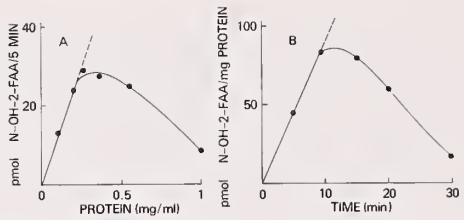
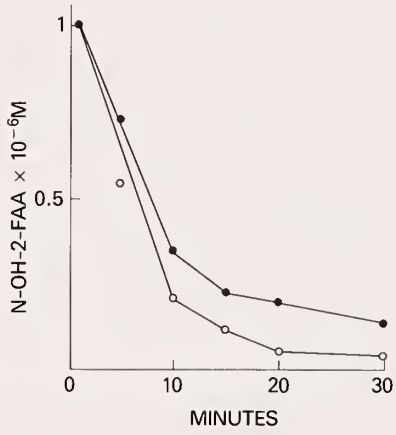


TEXT-FIGURE 2.—Guinea pig liver microsomal 2-FAA *N*-hydroxylase activity in the presence of 5×10^{-5} M paraoxon plus 0.1 M sodium fluoride as a function of protein concentration (A) and time of incubation (B). The concentration of 2-FAA was 5×10^{-6} M.

figure 3. Incubated in the presence of this subcellular preparation, *N*-OH-2-FAA at a concentration of 1×10^{-6} M rapidly disappears from the incubation medium; the half-life for such a transformation is in the order of 5 minutes. Guinea pig liver microsomes thus have an N-hydroxylating enzymatic system, which is highly active when 2-FA is used as a substrate. It is almost as active as the rat liver enzyme toward 2-FAA. The peculiarity of the guinea pig liver enzyme is its rapid conversion of the *N*-OH-metabolite to a



TEXT-FIGURE 3.—Metabolism of *N*-OH-2-FAA (1.10^{-6} M) by guinea pig liver microsomes (0.27 mg/ml) in the absence (○) or in the presence (●) of 0.1 M sodium fluoride.

yet unknown product. However, preliminary results seem to indicate that *N*-OH-2-FAA is efficiently transformed to 7-OH-2-FAA, a reaction which is not catalyzed by control rat liver microsomes (*11*). Such data, which need further investigation, would agree with the in vivo findings of high urinary excretion of 7-OH-2-FAA when guinea pigs are fed 2-FAA or *N*-OH-2-FAA (*1*).

REFERENCES

(*1*) MILLER EC, MILLER JA, ENOMOTO M: The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamster, and guinea pig. Cancer Res 24:2018–2031, 1964

(*2*) WEISBURGER EK, WEISBURGER JH: Chemistry, carcinogenicity and metabolism of 2-fluorenamine and related compounds. Adv Cancer Res 5:331–431, 1958

(*3*) BRIDENBACH AW, ARGUS MF: Attempted tumor induction in guinea pigs. Q J Fla Acad Sci 19:68–70, 1956

(*4*) IRVING CC: Enzymatic N-hydroxylation of the carcinogen 2-acetylaminofluorene-9-^{14}C in vitro. J Biol Chem 239: 1589–1596, 1964

(*5*) _____: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26: 1390–1396, 1966

(*6*) GUTMANN HR, BELL P: *N*-hydroxylation of arylamides by the rat and guinea pig. Evidence for substrate specificity and participation of cytochrome P_{450}. Biochim Biophys Acta 498:229–243, 1977

(*7*) RAZZOUK C, LHOEST G, ROBERFROID M, et al: Subnanogram estimation of the proximate carcinogen *N*-hydroxy-2-fluorenylacetamide by gas–liquid chromatography. Anal Biochem 83:194–203, 1977

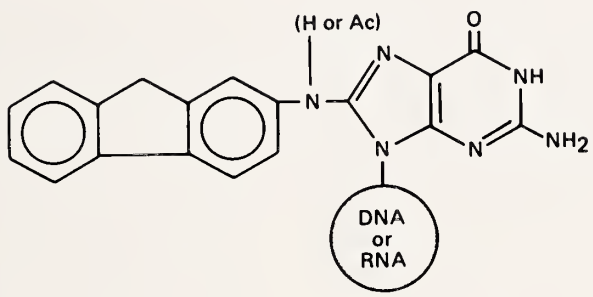
(*8*) RAZZOUK C, EVRARD E, LHOEST G, et al: Isothermal gas chromatography with wall-coated glass capillary columns, electron capture detection and a solid injector. II. Application to the assay of 2-fluorenylacetamide *N*-hydroxylase activity in a rat liver microsomal system. J Chromatog 16:103–109, 1978

(*9*) RAZZOUK C, MERCIER M, ROBERFROID M: Characterization of the guinea pig liver microsomal 2-fluorenylamine and 2-fluorenylamide *N*-hydroxylase. Cancer Lett 9:123–131, 1980

(*10*) _____: Induction, activation, and inhibition of hamster and rat liver microsomal arylamide and arylamine *N*-hydroxylase. Cancer Res 40:3540–3546, 1980

(*11*) _____: Biochemical basis for the resistance of guinea-pig and monkey to the carcinogenic effects of arylamines and arylamides. Xenobiotica 10:565–572, 1980

Session IV: Animal Studies: Adduct Formation



Chairman: Elizabeth C. Miller

Formation of *N*-2-Fluorenylhydroxylamine Adducts of DNA In Vivo and In Vitro and Some of Their Properties[1]

E. Kriek *and* J. G. Westra[2]

ABSTRACT—The major 2-fluorenylamine-DNA derivative formed in vivo in rat liver after application of *N*-2-fluorenylacetamide is *N*-(deoxyguanosin-8-yl)-2-fluorenylamine. This nucleoside, hydrolyzed under mild alkaline conditions with the opening of the imidazole ring, formed two pyrimidine derivatives which can be separated by Sephadex LH-20 column chromatography and thin-layer chromatography on silica. The hydrolysis reaction was catalyzed by metal ions and alkaline phosphatase from *Escherichia coli*.—Natl Cancer Inst Monogr 58: 139–142, 1981.

Arylamines form a group of compounds which can induce tumors in a variety of tissues in various species. In most species, with the exception of dogs, an equilibrium is established between acetylation of the arylamine and deacetylation of the N-acetyl derivative, which, under normal conditions, induces acetylation. Further studies in experimental animals have shown that the carcinogenicity of these compounds depends on their conversion to N-hydroxy derivatives. In this way, aryl acetamides are converted partially to aryl acethydroxamic acids, which can be further activated by various metabolic reactions, including esterification, deacetylation, transacetylation, and one-electron oxidation. Each of these reactions will be discussed by others in this Monograph. The reactive intermediates formed are capable of reacting nonenzymatically with nucleic acids and proteins to form covalent bonds. Chemical attack on nucleic acids, in particular DNA, is considered a necessary step in the initiation of the carcinogenic process.

The products of the reactions of several aryl acetamides with guanine in nucleic acids have now been characterized (*1*). For example, administration of the versatile carcinogen 2-FAA or its N-hydroxy derivative to rats leads to the formation of dGuo-2-FAA and 3-(deoxyguanosin-N^2-yl)-2-FAA (*1, 2*). The latter product is formed exclusively in DNA. These products differ appreciably in chemical properties, and Grunberger and Weinstein (*3*) have also detected large differences in conformation. In vivo, however, both compounds are minor components of rat liver DNA and represent not more than 35% of the total bound carcinogen. The other material does not include the N-acetyl group. Kriek (*4*) showed that *N*-hydroxy arylamines react readily with nucleic acids at a pH of 5 but not with nucleosides. When *N*-OH-2-FA reacts with DNA in vitro, only *N*-(guanin-8-yl)-2-FA is formed. This product was determined quantitatively by Westra and Visser (*5*) after hydrolysis in trifluoroacetic acid followed by high-pressure liquid chromatography of the hydrolysate. On the other hand, *N*-(guanin-8-yl)-2-FA was not detected in acid hydrolysates of rat liver DNA which had been treated with *N*-OH-[^{3}H]2-FAA. The following possibilities were considered:

1) Although dGuo-2-FA is not formed in vivo to an appreciable extent, the reaction does occur with other nucleic acids.

2) dGuo-2-FA is hydrolyzed to other products following its formation in situ.

3) dGuo-2-FA is hydrolyzed to other products during the enzymatic hydrolysis of DNA with DNase, venom phosphodiesterase, and alkaline phosphatase at a pH of 8–9.

On the basis of these considerations, we decided to study the chemistry of dGuo-2-FA in more detail, some of the results of which are presented here.

MATERIALS AND METHODS

Chemicals.—*N*-OH-[^{3}H]2-FA and dGuo-2-FAA were prepared according to the procedures described by Scribner and Naimy (*6*) and Kriek et al. (*7*). The deacetylated nucleoside dGuo-2-FA was prepared by the dissolution of dGuo-2-FAA in 25% ammonia and heating for 2 hours at 80° C while ammonia gas was bubbled continuously through the solution. After the reaction mixture is neutralized with 2 N acetic acid, dGuo-2-FA precipitates as a light yellow, flocculent product. It is further purified by Sephadex LH-20 chromatography. Under these conditions, little conversion to ureidopyrimidine derivatives takes place (*see* below). Calf thymus DNA (type I, highly polymerized), obtained from Sigma Chemical Co., St. Louis, Mo., was

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; dGuo-2-FAA = *N*-(deoxyguanosin-8-yl)-*N*-2-fluorenylacetamide; *N*-OH-2-FA = *N*-hydroxyfluorenamine; *N*-OH-[^{3}H]2-FAA = *N*-hydroxy-*N*-2-[G-^{3}H]-fluorenylacetamide; dGuo-2-FA = *N*-(deoxyguanosin-8-yl)-2-fluorenamine; pK_a = the negative logarithm to the base 10 of an acid dissociation constant; NMR = nuclear magnetic resonance; dR = deoxyribose moiety; m/z = mass divided by elementary charge.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Chemical Carcinogenesis Division, The Netherlands Cancer Institute, Antoni van Leeuwenhoekhuis, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands.

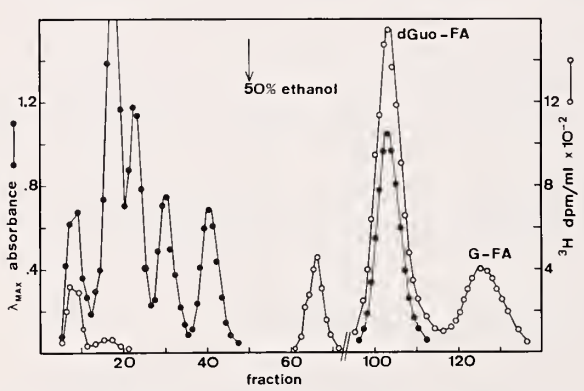
modified with *N*-OH[^{3}H]2-FA as described previously (*8*). All enzymes were obtained from Worthington Biochemical Corp., Freehold, N.J., except S_1 nuclease which was from Calbiochem, Los Angeles, Calif.

Enzymatic hydrolysis.—[^{3}H]2-FA-modified DNA was hydrolyzed either with a mixture of DNase, venom phosphodiesterase, and alkaline phosphatase as described in (*9*) or with S_1 nuclease and acid phosphatase. We denatured 5 mg of [^{3}H]2-FA-modified DNA in 0.005 M sodium acetate with a pH of 6.0 by heating in a boiling water bath for 10 minutes and then rapidly cooling it in ice. It was then incubated in 0.05 M sodium acetate buffer (pH 4.6) containing 0.05 M NaCl and 0.001 M zinc sulfate with S_1 nuclease (6,000 U) for 2 hours at 37° C. The pH was brought to 5.2 by the addition of 0.5 M sodium acetate, and the digestion was continued for 20 hours with 2 mg of acid phosphatase. The enzyme digests were chromatographed on columns of Sephadex LH-20 as described in the legends for text figures 1 and 2. The UV spectra were determined in a Zeiss PMQ II spectrophotometer equipped with a thermostated cuvette holder.

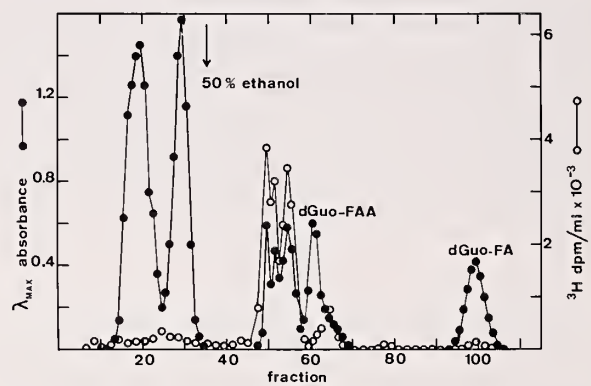
RESULTS

Complete hydrolysis of DNA modified with *N*-OH-[^{3}H]2-FA to 0.65% substituted bases was achieved with the S_1 nuclease and acid phosphatase procedure. The Sephadex LH-20 chromatography pattern illustrated in text-figure 1 shows that 55% of the DNA-bound radioactivity was co-chromatographed with the unlabeled dGuo-2-FA. Approximately 25% of the base *N*-(guanin-8-yl)-FA was eluted after the nucleoside, which indicated that depurination occurred during the enzymatic hydrolysis.

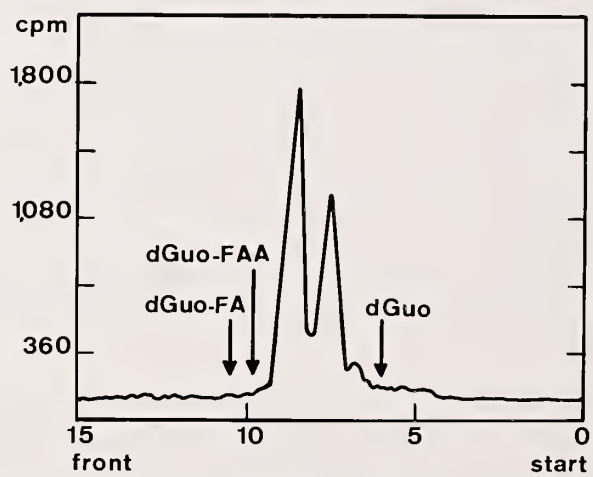
However, when the same DNA preparation was subjected to hydrolysis with DNase, venom phosphodiesterase, and alkaline phosphatase at pH 9, a completely different pattern was obtained on Sephadex LH-20 (text-fig. 2). Virtually no radioactivity traveled with the marker dGuo-2-FA, but most of the tritium activity was eluted *before* the marker compound dGuo-2-FAA. Further analysis of this material by thin-layer chromatography on silica F_{254} (obtained from E. Merck A. G., Darmstadt, Federal Republic of Germany) in butan-1-ol:acetic acid:water (50:11:25 by vol) revealed that 2 other compounds had been formed (text-fig. 3). The same products were formed when dGuo-2-FA was heated in 0.1 N NaOH at 75° C for 2 hours. Both compounds have identical UV spectra, which are characterized by a large decrease in absorbance at 320 nm (text-fig. 4). Both compounds have the same pK_a value



TEXT-FIGURE 1.—Sephadex LH-20 column (dimensions, 30×2 cm) chromatogram of a nucleoside hydrolysate obtained from [^{3}H]FA-modified DNA digested with S_1 nuclease and acid phosphatase as described in the Materials and Methods section. The column was eluted first with 200 ml 0.01 M ammonium carbonate for removal of unmodified nucleosides and then with 50% ethanol. Fractions of 5 ml were collected, and the radioactivity was measured in a Triton X-100-based scintillator. Unlabeled dGuo-2-FA was added to the hydrolysate. dpm = disintegrations/min.



TEXT-FIGURE 2.—Sephadex LH-20 column chromatogram of a nucleoside hydrolysate obtained from [^{3}H]-2-FA-modified DNA digested with DNase, venom phosphodiesterase, and alkaline phosphatase at pH 8–9 (*9*). Elution as described in text-figure 1. Unlabeled dGuo-2-FAA and dGuo-2-FA were added to the hydrolysate.



TEXT-FIGURE 3.—Silica F_{254} thin-layer chromatogram of the material from fractions 50–60 of the Sephadex LH-20 column chromatogram shown in text-figure 2. Solvent: butan-1-ol: acetic acid:water = 50:11:25 (by vol). The 20×10-cm plate was developed over a distance of 15 cm. Radioactivity scans were made with a Berthold LB 2722 thin-layer scanner. *Arrows* indicate the location of the unlabeled markers dGuo, dGuo-2-FAA, and dGuo-2-FA.

of approximately 8.5 as compared with 9.3 for dGuo-2-FA; the IR and [^{13}C]NMR spectra were also identical. The only difference observed between the [^{1}H]NMR spectra of both compounds was that indicating a different conformation of the dR. In the mass spectra of both compounds, the *m/z* value of 207.0688 of the base peak corresponded with fluorenyl isocyanate (calculated mass = 207.0684). This fragment is completely absent in the mass spectrum of dGuo-2-FA. Both deoxynucleosides are stable in 0.1 N HCl at 37° C and are hydrolyzed with difficulty at elevated temperatures. From the physical and chemical data, the structure was tentatively assigned as the ureidopyrimidine derivative illustrated in text-figure 5. A detailed structural analysis will be published elsewhere (*10*).

We followed the kinetics of the hydrolytic opening of the imidazole ring of dGuo-2-FA under various conditions by measuring the decrease of $A_{325\,nm}$. The reaction occurs at 37° C at slightly elevated pH values and is catalyzed by metal ions, particularly Mn^{2+}, and also by alkaline phosphatase. The first-order rate constants in 0.03 M sodium bicarbonate buffer (pH 9.5) at 37° C are presented in table 1. Venom phosphodiesterase, S_1 nuclease, and acid phosphatase did not catalyze the reaction. Arginase, found in many mammalian tissues, is especially abundant in liver and is activated by Mn^{2+} and Co^{2+} ions. Inasmuch as part of the structure of dGuo-2-FA formed by N_7, C_8, N_9, and the arylamine nitrogen resembles the structure of arginine, arginase was also tested for its ability to catalyze the hydrolytic opening of the imidazole ring. However, the addition of arginine to the reaction mixture did not increase the rate of the reaction as compared with the Mn^{2+}-maleate complex alone. Perhaps reactions of this type occur in vivo after the formation of *N*-(guanin-8-yl)-arylamine residues in nucleic acids.

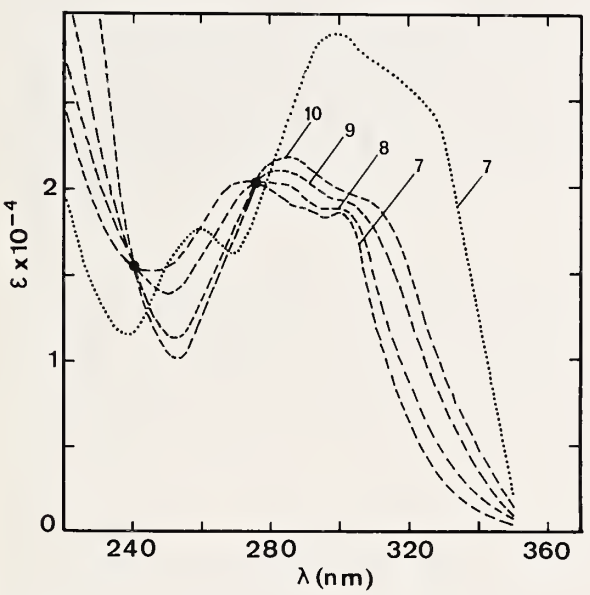
TABLE 1.—*First-order rate constants of the catalyzed hydrolysis of d-Guo-FA* [a]

Catalyst [b]	k_1, hr^{-1} [c]	$T_{½}$, hr	Relative rate
Solvent only	0.045 ± 0.004	15.4	1.0
Arginase	0.045 ± 0.004	15.4	1.0
0.16 mmol Mn^{2+}-maleate complex	0.795 ± 0.100	0.87	17.7
Arginase + 0.16 mmol Mn^{2+}-maleate	0.824 ± 0.084	0.84	18.3
0.16 mmol Mn^{2+}-EDTA complex	0.045 ± 0.004	15.4	1.0
0.16 mmol Mg^{2+}	0.125 ± 0.007	5.5	2.8
Alkaline phosphatase (*Escherichia coli*)	0.106 ± 0.006	6.5	2.4

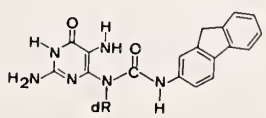
[a] The rate constants were calculated from the decrease of $A_{325\,nm}$ as a function of time; 0.04 mmol in 0.03 M sodium bicarbonate buffer at a pH of 9.5 at 37° C.

[b] Amount of enzymes used is 0.05 mg protein/ml.

[c] Values are given as standard error of the estimate.



TEXT-FIGURE 4.—UV absorption spectra of dGuo-2-FA (. . . . . .) and its alkaline hydrolysis product (- - - - -) in aqueous solutions of various pH. Isosbestic points (•) are at 240 and 276 nm.



TEXT-FIGURE 5.—Tentative structure of the alkaline hydrolysis products of dGuo-2-FA.

From the results obtained in this study we concluded that the enzymatic procedure for digestion of DNA at pH 9, in which DNase, venom phosphodiesterase, and alkaline phosphatase are required cannot be used for arylamine-modified DNA preparations which carry *N*-(guanin-8-yl)-arylamine residues when the modified deoxynucleosides are to be determined. The procedure in which S_1 nuclease and acid phosphatase are used will give a good yield of *N*-(deoxyguanosin-8-yl)-arylamine, but the corresponding *N*-acetyl derivative cannot be detected under these conditions because it is completely deacetylated in 24 hours at 37° C.

In addition to the acid stability of the ureidopyrimidine derivatives (text-fig. 5), these products also inhibit enzymatic digestion when present in polydeoxynucleotides. Because complete hydrolysis is required for their detection in vivo, we are now developing a procedure to improve the enzymatic digestion of DNA modified with the ureidopyrimidine derivatives of 2-FA.

REFERENCES

(*1*) KRIEK E, WESTRA JG: Metabolic activation of aromatic amines and amides and interaction with nucleic acids. In Chemical Carcinogens and DNA (Grover PL, ed), vol II. Boca Raton, Fla.: CRC Press, 1979, pp 1–28

(*2*) KRIEK E: Carcinogenesis by aromatic amines. Biochim Biophys Acta 355:177–203, 1974

(*3*) GRUNBERGER D, WEINSTEIN IB: Conformational changes in

nucleic acids modified by chemical carcinogens. *In* Chemical Carcinogens and DNA (Grover PL, ed), vol II. Boca Raton, Fla.: CRC Press, 1979, pp 59–93

(*4*) KRIEK E: On the interaction of *N*-2-fluorenylhydroxylamine with nucleic acids in vitro. Biochem Biophys Res Commun 20:793–799, 1965

(*5*) WESTRA JG, VISSER A: Quantitative analysis of *N*-(guanin-8-yl)-*N*-acetyl-2-aminofluorene and *N*-(guanin-8-yl)-*N*-2-aminofluorene in modified DNA by hydrolysis in trifluoroacetic acid and high pressure liquid chromatography. Cancer Lett 8:155–162, 1979

(*6*) SCRIBNER JD, NAIMY NK: Reaction of esters of *N*-hydroxy-2-acetamidophenanthrene with cellular nucleophiles and the formation of free radicals upon decomposition of *N*-acetoxy-*N*-arylacetamides. Cancer Res 33:1159–1164, 1973

(*7*) KRIEK E, MILLER JA, JUHL U, et al: 8-(*N*-2-Fluorenylacetamido) guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide in neutral solution. Biochemistry 6:177–182, 1967

(*8*) KRIEK E: Effect of pH on the ratio of substitution products in DNA after reaction with the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. Cancer Lett 7:141–146, 1979

(*9*) _____: Binding of *N*-2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids in vivo. Chem Biol Interact 1:3–17, 1969

(*10*) KRIEK E, WESTRA JG: Structural identification of the pyrimidine derivatives formed from *N*-(deoxyguanosin-8-yl)-2-aminofluorene in aqueous solution at alkaline pH. Carcinogenesis 1:459–468, 1980

Formation of DNA Adducts by the Carcinogen *N*-Hydroxy-2-naphthylamine[1]

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ABSTRACT—The probable ultimate urinary bladder carcinogen, *N*-hydroxy-2-naphthylamine (*N*-OH-2-NA), reacted with nucleic acids and proteins under mildly acidic conditions (pH 5) to form covalently bound derivatives. The extent of reaction was in the order: Polyguanylic acid > DNA ≈ protein > rRNA > tRNA > polyadenylic acid ≈ polyuridylic acid > polycytidylic acid. At pH 7, appreciable reaction occurred only with protein. Enzymatic hydrolyses of the DNA, which contained 1.5 naphthyl residues/1,000 nucleotides, yielded 3 nucleoside–arylamine adducts. From chemical, UV, nuclear magnetic resonance, and mass spectrometric analyses, the adducts were identified as 1-(deoxyguanosin-N^2-yl)-2-NA, 1-(deoxyadenosin-N^6-yl)-2-NA, and a purine ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA, tentatively identified as 1-[5-(2-6-diamino-4-oxopyrimidinyl-N^6-deoxyriboside)]-2-(2-naphthyl)urea. Preliminary experiments with a dog given [^{3}H]2-NA suggested the presence of these adducts in vivo. The properties of adducts derived from *N*-OH-1-NA and *N*-OH-2-NA and their possible roles in the initiation of carcinogenesis are discussed.—Natl Cancer Inst Monogr 58: 143–152, 1981.

The conversion of certain *N*-OH arylamines to arylnitrenium ion or carbocation electrophiles under acidic conditions was first proposed by Yugawa (*1*) and Ingold and co-workers (*2*) in the 1950's and by Boyland et al. (*3*) and Kriek (*4*) in the 1960's. In the latter study, the N-OH derivatives of 2-FA, 2-NA, and 4-aminobiphenyl were incubated with DNA and RNA at a mildly acidic pH (4.0–6.0). Upon reisolation of the nucleic acids, decreased guanylic acid content was noted as well as increased UV absorbance at 320 nm, which suggested the presence of covalent nucleic acid–carcinogen adducts. This hypothesis was confirmed for [ring-^{3}H]*N*-OH-2-FA when it reacted with DNA to yield a product with a ^{3}H label that could not be removed by extensive dialysis or chromatography (*5*). The major FA–DNA derivative formed in this reaction was then identified as *N*-dG-8-yl)-2-FA (*6, 7*) and was identical to the major adduct formed from *N*-OH-2-acetylaminofluorene in vivo in rat liver (*8*) and in vitro by the action of hepatic *N,O*-acyltransferase (*7*).

Inasmuch as *N*-OH arylamines are known urinary metabolites of arylamine bladder carcinogens, their conversion to reactive electrophiles in the normally acidic bladder lumen of humans and dogs and the subsequent binding of these electrophiles to bladder epithelial DNA have been proposed as critical steps in arylamine-induced bladder carcinogenesis (*9–11*). We (*9*) showed that [^{3}H] *N*-OH-1-NA and [^{3}H]*N*-OH-2-NA reacted covalently with DNA when incubated at pH 5–6; the extent of substitution (carcinogen residues/nucleotide) was approximately 1/100 and 1/1,000, respectively.

Recently, the DNA adducts formed by reaction with *N*-OH-1-NA in vitro were identified as *N*-(dG-O^6-yl)-1-NA and 2-(dG-O^6-yl)-1-NA and its decomposition product (*12*). Because O^6-substituted dG adducts represent potential mispairing lesions (*13, 14*), the formation of these 1-NA–DNA adducts in vivo may constitute the initiation step in *N*-OH-1-NA carcinogenesis. Although *N*-OH-1-NA is strongly carcinogenic at the sites of application (*15, 16*) and reacts readily with DNA in vitro (*12*), the parent 1-NA, a suspected human carcinogen (*17, 18*), has not proved to be carcinogenic in animal bioassays (*18, 19*). The poor ability of the test species to N-oxidize the parent compound (*20, 21*) and the presence of detoxification mechanisms (*22*) have been cited as possible explanations.

In contrast, 2-NA is carcinogenic to the bladders of humans, dogs, guinea pigs, rabbits, monkeys, hamsters, and rats, and to the livers of mice (*19*). Considerably more carcinogenic than 2-NA (*15, 16, 23*), *N*-OH-2-NA also induces transitional cell carcinomas of the dog urothelium when directly instilled into the bladder (*16*) and is believed to represent the proximate or ultimate form of the carcinogen. Dr. E. C. Miller et al. (unpublished data) have shown that *N*-OH-2-NA is only weakly carcinogenic at subcutaneous sites of application in the rat (10% sarcoma incidence at 15 mo), whereas *N*-OH-1-NA is strongly carcino-

Abbreviations: 2-FA = 2-fluorenamine; 2-NA = 2-naphthylamine; *N*-OH-1-NA = *N*-hydroxy-1-naphthylamine; *N*-OH-2-NA = *N*-hydroxy-2-naphthylamine; Bis-Tris = bis(2-hydroxyethyl)-tris(hydroxymethyl)-aminomethane base; dG = deoxyguanosin; dA = deoxyadenosine; d_6 = deuterated; NaOD = sodium deuteroxide; DMSO = dimethyl sulfoxide; D_2O = deuterium oxide; dR = deoxyribose; HPLC = high-pressure liquid chromatography; ppm = parts/million; NMR = nuclear magnetic resonance; *m/z* = mass divided by elementary charge; TMS = tetramethylsilane.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979. This paper has been published in part in *Carcinogenesis* 1:139–150, 1980; 2:467–470, 1981.

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genic under these conditions and yields a 100% incidence of sarcomas at 12 months.

Relating these results to specific mechanisms of arylamine carcinogenicity requires studies on the identity of the DNA adducts formed from 1- and 2-NA and from their N-OH metabolites and others on the persistence and repair of these lesions. In this report, the carcinogen–DNA adducts formed by reacting [^{3}H]*N*-OH-2-NA with DNA in vitro at pH 5 were isolated and characterized. The presence of these adducts in vivo in dog liver and bladder epithelium after administration of [^{3}H]2-NA was also investigated.

MATERIALS AND METHODS

Materials.—Calf thymus DNA (type I); tRNA (type III); polyadenylic, polyguanylic, polycytidylic, and polyuridylic acids; human serum albumin (fraction V); deoxyribonuclease I (type DNCl); phosphodiesterase I (type II); alkaline phosphatase (type III-S); deoxyribonucleosides; Sephadex G-15; and Bis–Tris and Tris bases were obtained from the Sigma Chemical Co. (St. Louis, Mo.). The rRNA was prepared from rat liver (*24*); [5,6,7,8-^{3}H]*N*-OH-2-NA[5] (10–100 mCi/mol), *N*-OH-2-NA, and [5,6,7,8-^{3}H]2-NA[5] (100 mCi/mmol) were obtained from Midwest Research Institute (Kansas City, Mo.). We purchased [U-^{14}C]deoxyadenosine, [U-^{14}C]dG, [U-^{14}C]deoxycytidine, and [2-^{14}C]-thymidine from Amersham Corporation (Arlington Heights, Ill.), and the d_6-DMSO, NaOD, and D_2O were obtained from Merck and Co. (St. Louis, Mo.). N^2-Ethyldeoxyguanosine, *N*-1-methyldeoxyadenosine, *N*-3-methyladenine, O^6-methyldeoxyguanosine, and N^6-methyladenosine were provided by D. Beranek of the National Center for Toxicological Research.

Reactivity of [^{3}H]N-OH-2-NA with nucleic acids, protein, and deoxyribonucleosides.—For determinations of the covalent binding of [^{3}H]*N*-OH-2-NA to nucleic acids and protein, incubations were done under argon at 37° C for 4 hours in 10 mM potassium citrate buffer (pH 5 or 7) containing 0.1 mM EDTA, 5 mg nucleic acid or protein/ml, and 1 mM [^{3}H]*N*-OH-2-NA (41 mCi/mmol). Nucleic acids and protein were isolated and bound ^{3}H was estimated as described in (*12, 25*). To determine the reaction products of *N*-OH-2-NA with deoxyribonucleosides, we used either 0.5 mM [^{3}H]*N*-OH-2-NA and 2 mM unlabeled deoxyribonucleosides or 10 mM *N*-OH-2-NA and 1 mM [^{14}C]-labeled deoxyribonucleosides in the incubations. We analyzed aliquots by HPLC, collected fractions corresponding to the retention times of *N*-OH-2-NA–DNA adducts I, II, and III, and determined their radioactivity.

Preparation and isolation of N-OH-2-NA-DNA adducts in vitro.—One mM [^{3}H]*N*-OH-2-NA was incubated with 5 mg DNA/ml at pH 5 in 10 mM potassium citrate buffer–0.1 mM EDTA for 4 hours under argon (total volume = 100 ml). The DNA was reisolated (*12, 24*) and dissolved in 250 ml of 5 mM Bis-Tris–HCl buffer (pH 7.1)–5 mM $MgCl_2$. Enzymatic hydrolysis of the DNA was performed by addition of deoxyribonuclease I (5 ml of 10 mg protein/ml in 0.15 M NaCl) and subsequent incubation at 37° C for 3 hours under argon. The mixture was then adjusted to pH 9 by the addition of 8–10 ml of 1 M Tris base; 500 U of alkaline phosphatase and 20 U of venom phosphodiesterase were added (final pH = 8.5–8.8). The mixture was flushed with argon and incubated at 37° C for 8 hours. Six grams ammonium sulfate was added and the hydrolysate was extracted twice with equal volumes of water-saturated *n*-butanol (redistilled). The butanol extracts, which contained 90–95% of the [^{3}H]*N*-OH-2-NA–nucleoside adducts, were evaporated at 35° C under reduced pressure. The residue was dissolved in 5–10 ml of 50% methanol, applied to a Sephadex G-15 column (2.5 × 28 cm), and eluted with 10 mM potassium phosphate buffer (pH 7.4) containing 50% methanol.

The *N*-OH-2-NA–nucleoside adduct fractions that eluted from the Sephadex column were concentrated tenfold under reduced pressure at 35° C and extracted with an equal volume of *n*-butanol. The butanol extract containing the adducts was evaporated to dryness, the residue dissolved in 2–3 ml of 50% methanol, and the adducts purified by HPLC on a μBondapak C_{18} reversed-phase column (0.39 × 30 cm) purchased from Waters Associates, Inc. (Milford, Mass.). The fractions were collected, flushed with argon, evaporated to dryness, and stored at −70° C for subsequent analyses. For comparisons of adduct stability, each *N*-OH-2-NA–nucleoside adduct (25 μM) was incubated at pH 1 (0.1 N HCl), pH 7 (10 mM potassium citrate buffer), or pH 13 (0.1 N NaOH) under argon at 37° C for 4 hours. Recovery was determined by HPLC by integration of UV-absorbing peaks.

Analysis of dog liver and bladder macromolecules after [^{3}H]2-NA administration.—The [^{3}H]2-NA (100 mCi/mmol; 72 mg) was given orally in a gelatin capsule to a 9-kg adult male beagle dog obtained from Marshall Research Animals Inc. (North Rose, N.Y.). After 24 hours, the animal was euthanized by (T-61 Euthanasia Solution ordered from the Taylor Pharmacal Co. (Decatur, Ill.), and the liver and bladder were excised. Bladder epithelium was separated mechanically from the muscle. The DNA, RNA, and protein from liver and bladder epithelum were isolated by extraction and hydroxyapatite chromatography as described in (*25*); DNA was hydrolyzed as described earlier, and nucleoside adducts were analyzed by HPLC.

Instrumentation.—Sephadex column fractions were collected at 5° C on an ISCO (Lincoln, Nebr.) Golden Retriever fractionator equipped with a UA-5 absorbance monitor. We performed the HPLC on a Waters Associates, Inc. Model 204 chromatograph equipped with an additional Model 6000A pump, a Model 440 dual channel (254 and 313 nm) absorbance monitor, a Model 660 Solvent programmer, and a Hewlett-Packard (Avondale, Pa.) 3380A Recording Integrator. Radioactivity was determined in Scintisol purchased from Isolab, Inc. (Akron, Ohio) on a G. D. Searle & Co. (Des Plaines, Ill.) Mark III scintillation spectrometer. The UV spectra were recorded on a Cary (Palo Alto, Calif.) 219 recording spectrophotometer. Fourier transform proton NMR spectra were obtained on a 270- or 500-MHz spectrometer with an Aspect

[5] From ^{3}H–NMR spectra, 40% of the ^{3}H was at H-5, 40% at H-8, 15% at H-7, and 5% at H-6. Spectra were obtained through the courtesy of the State University of New York at Stony Brook/National Institutes of Health-sponsored nuclear magnetic resonance facility.

2000 computer from Bruker Instruments, Inc. (Billerica, Mass.). Field desorption mass spectra and high-resolution, electron impact mass spectra were recorded by direct probe on a modified Kratos-AEI (Manchester, U.K.) MS-902 mass spectrometer.

RESULTS

Reactivity of [^{3}H]*N*-Hydroxy-2-naphthylamine With Nucleic Acids, Protein, and Deoxyribonucleosides

The acidic pH-dependent conversion of *N*-OH-2-NA to an electrophilic arylnitrenium ion-carbocation that bound covalently to DNA and RNA was reported in (*9*). In table 1, additional studies on the reactivity of *N*-OH-2-NA with macromolecules at pH 5 and 7 are presented. The extent of reaction at pH 5 was as follows in descending order: Polyguanylic acid > DNA ≈ protein > rRNA > tRNA > polyadenylic acid ≈ polyuridylic acid > polycytidylic acid. At pH 7, the reaction with nucleic acids was markedly reduced; however, appreciable binding was observed with protein. This may be due to the slow oxidative decomposition of *N*-OH-2-NA, which occurs at pH 7 but not pH 5 (*9*), to yield nitroso derivatives that would be expected to react with protein sulfhydryl groups (*26*). The incubation of 1 mM *N*-OH-2-NA with 5 mg DNA/ml (corresponding to 15 mM deoxyribonucleotide) at pH 5 yielded approximately 1.5 arylamine residues bound/1,000 nucleotides; about 2.5% of *N*-OH-2-NA added became bound to the DNA.

Under similar incubation conditions with 10 mM *N*-OH-2-NA and 1 mM [^{14}C]-labeled dG, deoxyadenosine, deoxycytidine, and thymidine, the formation of *N*-OH-2-NA-nucleoside adducts was not detected (*see* "Materials and Methods"). The limit of detection in this assay was only 0.5% of the deoxyribonucleoside added. Inasmuch as only 0.3% of the deoxyribonucleoside bases in DNA become modified under comparable conditions, the results of the assay were inconclusive. Therefore, 0.5 mM [^{3}H]*N*-OH-2-NA was similarly incubated with 2 mM unlabeled deoxyribonucleosides; again no reaction was observed. Under these conditions, the limit of detection was 1% of the *N*-OH-2-NA added, but under comparable conditions, 3% of the *N*-OH-2-NA added reacted with DNA. Thus if *N*-OH-2-NA reacts with nucleosides, the extent of reaction is ≤ one-third the reaction obtained with DNA. Thus like *N*-OH-1-NA (*12*), *N*-OH-2-NA apparently does not react appreciably with deoxyribonucleoside monomers. Furthermore, in competition experiments, the addition of 1 mM deoxyribonucleosides, glutathione, or ascorbic acid to *N*-OH-2-NA–DNA incubations failed to alter significantly (± 10%) covalent binding to DNA.

TABLE 1.—*Reactivity of* [^{3}*H*]N-*OH-2-NA with nucleic acids and protein*[a]

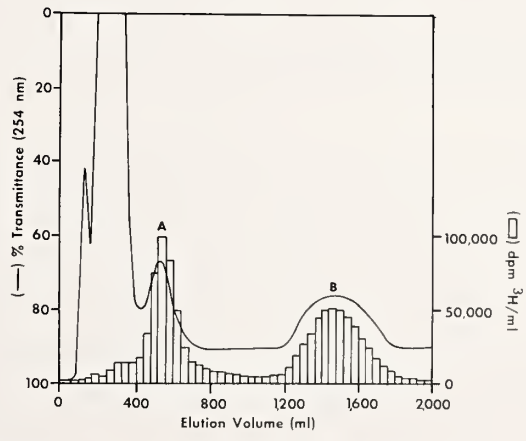
Carcinogen binding to:	nmol [^{3}H]*N*-OH-2-NA bound/mg polymer	
	pH 5.0	pH 7.0
DNA	5.1	0.4
rRNA	3.7	0.2
tRNA	1.0	0.2
Polyadenylic acid	0.3	0.1
Polyguanylic acid	10.9	0.4
Polycytidylic acid	0.1	0.1
Polyuridylic acid	0.3	0.1
Serum albumin	4.9	8.4

[a] Incubation mixtures contained 10 mM potassium citrate (pH 5.0 or 7.0), 0.1 mM EDTA, 5 mg polymer/ml, and 1.0 mM [^{3}H]*N*-OH-2-NA (41 mCi/mmol). Incubation conditions were: 37 ° C; 4 hr; argon atmosphere.

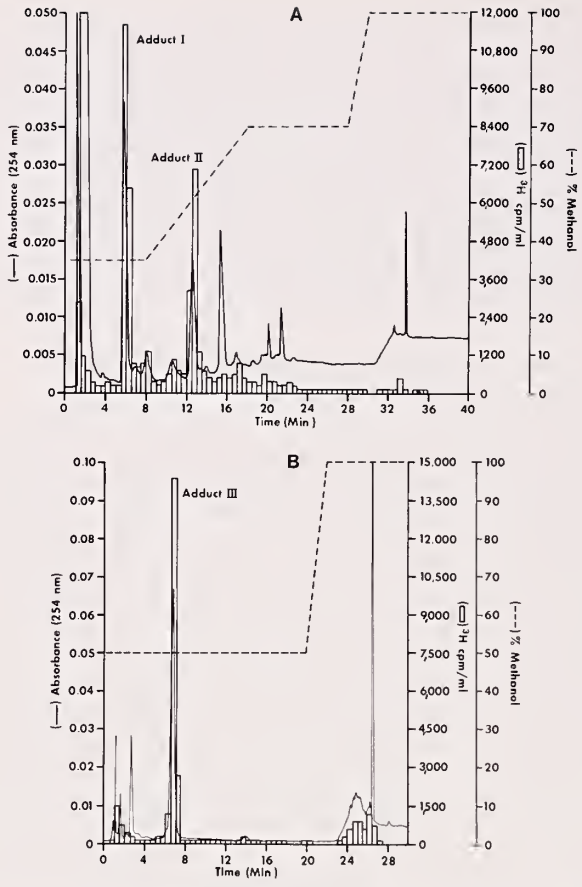
Isolation of the Adducts Formed by the In Vitro Reaction of *N*-Hydroxy-2-naphthylamine With DNA at Acidic pH

The reaction of the carcinogen *N*-OH-1-NA selectively with DNA to yield O^6-guanine-substituted adducts prompted an investigation of the identity of DNA adducts formed from *N*-OH-2-NA. After reaction of [^{3}H]*N*-OH-2-NA with DNA at pH 5 for 4 hours, the DNA was reisolated, purified, and enzymatically hydrolyzed to deoxyribonucleosides (*see* "Materials and Methods"). We recovered 90–95% of the radioactivity present in the hydrolysate by *n*-butanol extractions that were then applied to a Sephadex G-15 column. Subsequent chromatography yielded two UV-absorbing ^{3}H-containing peaks, labeled A and B, that eluted at 400–700 and 1,200–1,800 ml, respectively (*see* text-fig. 1); these peaks represented over 95% of the ^{3}H applied to the column.

Fractions A and B were each concentrated, extracted with butanol, evaporated, and reconstituted in aqueous methanol; 300- to 500-ul aliquots were then further purified by HPLC. As shown in text-figure 2, fraction A contained two major UV-absorbing ^{3}H-containing peaks, labeled adducts I and II, which eluted at 6 and 13 minutes, respectively. Fraction B (text-fig. 2) contained a single radioactive, UV-absorbing peak, labeled adduct III, which eluted at 6.5 minutes with a different solvent program. Ad-



TEXT-FIGURE 1.—Elution profile from a Sephadex G-15 column of an enzymatic hydrolysate of DNA that had been reacted with [^{3}H]*N*-OH-2-NA. Eluant was 10 mM potassium phosphate buffer (pH 7.4) and 50% methanol. *Fractions* labeled A and B were each pooled and purified by HPLC. dpm = disintegrations/min.



TEXT-FIGURE 2.—HPLC elution profile of *fractions* A and B from the Sephadex G-15 column. The position of adducts I, II, and III and the solvent program are indicated on the text-figure; the flow rate was 2 ml/min. cpm = counts/min.

ducts I, II, and III accounted for 30, 15, and 50%, respectively, of the radioactivity eluted from the Sephadex column.

Identification of Adduct I as 1-(Deoxyguanosin-N^2-yl)-2-naphthylamine

Analysis of a silylated derivative of adduct I by high-resolution electron impact mass spectrometry indicated that it was a tetrakis(trimethylsilyl) deoxyguanosinyl-naphthylamine adduct (table 2). Because dG and 2-NA together contain 5 available sites for silylation, the substitution of 4 silyl groups onto the adduct indicated that one of these sites was modified. This modification suggested several possible structures, i.e., an O^6-substituted derivative through the naphthyl nitrogen, or an N^2- or N-1-substituted adduct through a naphthyl carbon. From a determination of its solvent partitioning characteristics at pH's 1–13 by the method of Moore and Koreeda (*27*), adduct I was ionized at both acid and alkaline pH (text-fig. 3), and its UV spectra at pH's 1, 7, and 13 were distinctly different from each other (text-fig. 4) and similar to spectra obtained by an admixture of equimolar amounts of 2-NA and dG. These data rule out substitution at N-1 or O^6 of guanine and suggest that adduct I is an N^2-dG-substituted derivative.

TABLE 2.—*High resolution mass spectral data for silylated N-OH-2-NA-DNA* [a]

Adducts	*m/z* [b]	Elemental formula [error in ppm]	Assignment
I	696.3098	$C_{20}H_{16}O_4N_6$ $(TMS)_4$ [−4.26]	$(TMS)_4$-dG-NA (m^+)
	681.2832	$C_{20}H_{16}O_4N_6$ $(TMS)_3(DMS)_1$ [−8.88] [c]	m^+-CH_3
	508.2247	$C_{15}H_9O_1N_6$ $(TMS)_3$ [−2.28]	$(TMS)_5$ G-NA
	436.1860	$C_{15}H_{10}O_1N_6$ $(TMS)_2$ [−0.77]	m^+-dR
	421.1623	$C_{15}H_{10}O_1N_6$ $(TMS)_1(DMS)_1$ [−1.20]	m^+-dR-CH_3
	364.1460	$C_{15}H_{11}O_1N_6$ $(TMS)_1$ [−2.14]	m^+-dR-TMS
II	536.2402	$C_{20}H_{18}O_3N_6$ $(TMS)_2$ [+2.75]	$(TMS)_2$-dA-NA (m^+)
	521.2158	$C_{20}H_{18}O_3N_6$ $(TMS)_1$ $(DMS)_1$ [+1.00]	m^+-CH_3
	286.0964	?	?
	276.1122	$C_{15}H_{12}N_6$ [−0.73]	m^+-dR
	260.0929	$C_{15}H_{10}N_5$ [−1.89]	m^+-dR-NH_2
	249.1010	$C_{14}H_{11}N_5$ [−1.84]	m^+-dR-HCN [c]
III [d, e]	768.3479	$C_{20}H_{15}O_4N_6$ $(TMS)_5$ [−5.70]	$(TMS)_5$-dG-NA (m^+)
	696.3111	$C_{20}H_{16}O_4N_6$ $(TMS)_4$ [−2.29]	$(TMS)_4$-dG-NA (m'^+)
	508.2276	$C_{15}H_9O_1N_6$ $(TMS)_3$ [+3.46]	m^+-dR
	493.2028	$C_{15}H_9O_1N_6$ $(TMS)_2(DMS)_1$ [+0.95]	m^+-dR-CH_3
	436.1865	$C_{15}H_{10}O_1N_6$ $(TMS)_2$ [+0.39]	m'^+-dR
	421.1640	$C_{15}H_{10}O_1N_6$ $(TMS)_1(DMS)_1$ (+2.63]	m'^+-dR-CH_3

[a] Adducts (ca. 5 μg) were silylated with 200 μl bis(trimethylsilyl)-trifluoroacetamide:pyridine (4:1), 70° C, 1.5 hr.

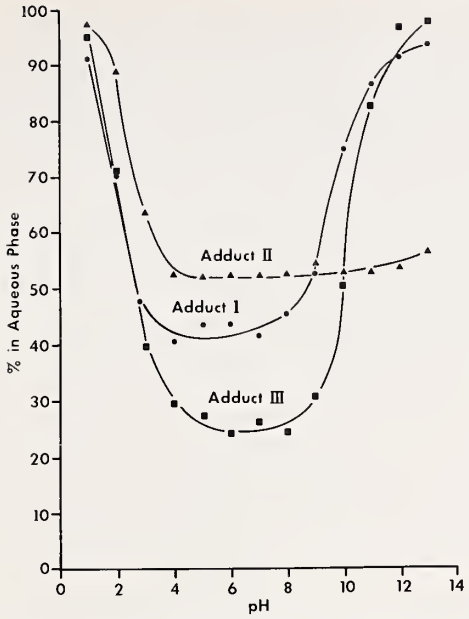
[b] Only major fragments with $m/z > 200$ are listed.

[c] DMS = dimethlysilyl; HCN = hydrocyanic acid.

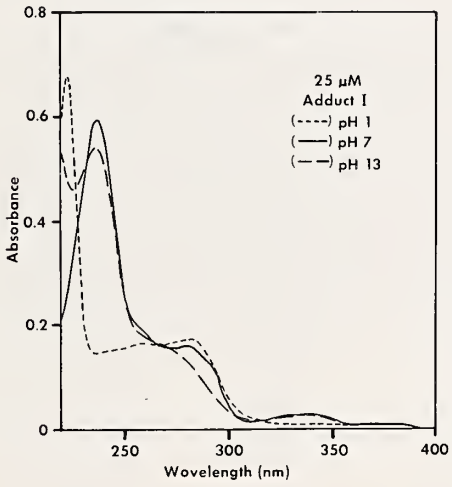
[d] Decomposition product with m/z 634, 624, 446, 431, 374, 364, 359, 347, 331, 275, 223, and 208 was formed upon analysis.

[e] Structure was confirmed by low resolution field desorption mass spectrum on underivatized sample (m^+ = 408).

Final structural elucidation was obtained from analysis of proton NMR data obtained at 270 and 500 MHz. The assignments were based on comparisons of adduct I spectra with spectra of 2-NA (text-fig. 5, table 3), dG [(*28*); unpublished data], N^2-ethyl-dG (unpublished data), 3-dG-N^2-yl)-*N*-methyl-4-aminoazobenzene (*29*), and with spectra previously reported for 3-(dG-N^2-yl)-*N*-acetyl-2-FA (*30*). For adduct I (text-fig. 6, table 4), the chemical shift and coupling pattern of the aromatic region revealed the presence of the H-3 through H-8 protons of the naphthyl ring. The assignment of naphthyl N^2-H_2 to the two exchangeable protons at 5.35 ppm was based on the similarity between their chemical shift and that of the 2-amino



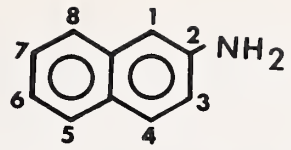
TEXT-FIGURE 3.—Partition of 1 μM adduct I, adduct II, and adduct III between aqueous buffers, pH 1–10 (*27*) and 50% *n*-butanol in ether, 50% *n*-butanol in ether, and 15% *n*-butanol in ether, respectively.



TEXT-FIGURE 4.—UV spectra of adduct I in 0.1 N HCl (- - -), 10 mM potassium citrate at pH 7 (——), and 0.1 N NaOH (– – –).

protons of 2-NA (5.38 ppm). The only naphthyl proton absent was H-1, which indicated that the 1-naphthyl carbon was the site of substitution. Assignment of the naphthyl H-1 to the resonance at 7.91 ppm was excluded because meta coupling for this peak and for naphthyl H-3 was not observed. The single at 7.91 ppm was thus assigned as the

[6] Resonances from N¹-H of guanine compounds as well as the 3′ and 5′ hydroxyl protons of deoxyribosides are often obscured due to exchange broadening if samples are not sufficiently dry and free of salts.



TEXT-FIGURE 5.—Structural formula for 2-NA.

TABLE 3.—*Proton NMR (270 MHz) data for 2-NA, including chemical shift, number of protons, and assignments*[a]

Chemical shift, ppm from TMS	Multiplicity (No. of protons)	Assignments[b]
7.62	D[c] } (2)	H-5
7.59	D[c] }	H-4
7.49	D[d] (1)	H-8
7.27	T (1)	H-7
7.08	T (1)	H-6
6.93	D[e] (1)	H-3
6.81	S (1)	H-1
5.38[f]	S (2)	N^2-H_2

[a] Five mg of 2-NA was dissolved in 0.3 ml d_6-DMSO. T = triplet; D = doublet; S = singlet. The S, D, and T nomenclature for the aromatic protons is used to describe the overall appearance of the peaks which arise from the ortho coupling constants.

[b] Assignments were determined from homonuclear decoupling experiments and nuclear Overhauser effects.

[c] J (coupling constant) was not determined due to resonance overlap.

[d] J_{ortho} = 8 Hz. Ortho = ortho coupling constant.

[e] J_{ortho} = 9 Hz.

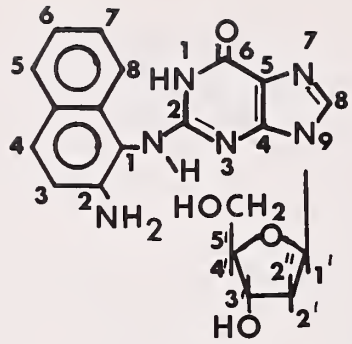
[f] This singlet is exchangeable with D_2O.

guanine C-8 proton as its shift was in close agreement with C-8-H (7.93 ppm) of dG. The single exchangeable proton at 8.19 ppm may represent N^2-H or N-1-H of dG; however, inasmuch as completely dry samples could not be prepared without substantial adduct decomposition, detection of both NH's was not achieved, and this peak could not be accurately assigned.[6] Nevertheless, the lack of a resonance assignable to the two protons at the N^2 amino group of dG (6.47 ppm) indicated substitution at this position. Thus these data are uniquely consistent with the identity of adduct I as 1-(dG-*N*²-yl)-2-NA.

Identification of Adduct II as 1-(Deoxyadenosin-*N*⁶-yl)-2-naphthylamine

Mass spectral analyses (table 2) of a silylated derivative of adduct II were consistent with a bis(trimethylsilyl)-deoxyadenosinyl-naphthylamine structure. From detailed analyses of fragments, we observed that only the 3′- and 5′-hydroxyl groups of the dR were silylated. Thus the site of substitution was unclear and involvement of either the naphthyl nitrogen and/or the adenine N^6 position was suggested. From its solvent partitioning at different pH's, the lack of an ionizable species at an alkaline pH was observed (text-fig. 3). Its UV spectra (text-fig. 7) at pH's 1, 7, and 13 were also characteristic of a deoxyadenosine derivative and were similar to spectra obtained by admixture of equimolar amounts of 2-NA and deoxyadenosine.

Structural characterization was achieved from 270 and



TEXT-FIGURE 6.—Structural formula for adduct I.

TABLE 4.—*The 270 and 500 MHz NMR data of 2-NA adduct I*[a]

Chemical shift, ppm from TMS	Multiplicity (No. of protons)	Assignments
8.19[b]	S (1)	N^1-H or N^2H (guanine)
7.91	S (1)	H-8 (guanine)
7.71	D[c] } (2)	H-8, 5, or 4 (naphthyl)
7.64	D[c] }	H-5, 8, or 4 (naphthyl)
7.55	D[d] (1)	H-4, 5, or 8 (naphthyl)
7.35	T (1)	H-7 (naphthyl)
7.15	T (1)	H-6 (naphthyl)
7.12	D[d, e] (1)	H-3 (naphthyl)
5.87	M (1)	H-1′ (dR)
5.35[b]	S (2)	N^2-H_2 (naphthyl)
4.09	M (1)	H-3′ (dR)
2.9–3.8	—	H_2O
2.51	—	d_5-DMSO
2.42	M (1)	H-2′ or 2″ (dR)
1.99	M (1)	H-2″ or 2′ (dR)

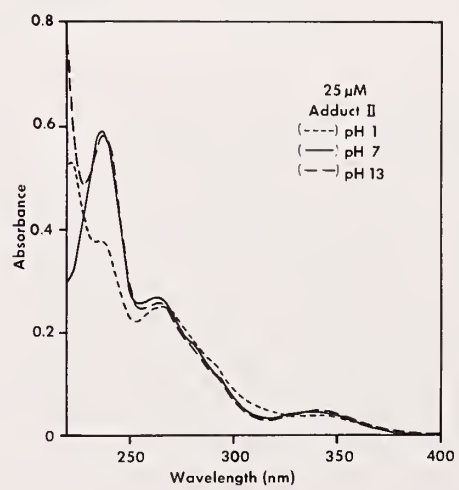
[a] We dissolved 50- to 200-μg samples in 0.1–0.25 ml of d_6-DMSO under N_2. The following protons were unresolved: OH-3′ (dR), OH-5′ (dR), N^1-H or N^2-H (guanine) H-4′ (dR), H-5′ (dR), and H-5″ (dR). M = multiplet; T = triplet; D = doublet; S = singlet.

[b] This singlet is exchangeable with D_2O.

[c] J was not determined due to resonance overlap.

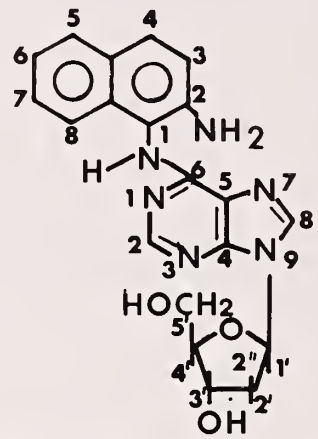
[d] J_{ortho} = 8.5 Hz.

[e] No meta coupling was detected at 500 MHz.



TEXT-FIGURE 7.—UV spectra of adduct II in 0.1 N HCl (- - -), 10 mM potassium citrate at pH 7 (——), and 0.1 N NaOH (– – –).

500 MHz proton NMR spectroscopy. Assignments were based on comparisons with NMR spectra of 2-NA, deoxyadenosine [(*28*); Kadlubar et al: Unpublished data], 1-methyldeoxyadenosine, 3-methyladenine, and N^6-methyladenosine (unpublished data). As summarized in text-figure 8 and table 5, the data indicated a 1-naphthyl-substituted structure for reasons similar to those discussed for adduct I. In addition, the two nonexchangeable singlets at 8.46 and 8.05 ppm had chemical shifts that were characteristic of adenyl H-8 and H-2 protons. Both protons also exhibited unusual line broadening at 270 MHz. Line broadening of the adenyl H-2 was also observed with the model compound, 6-methyladenosine, under similar ex-



TEXT-FIGURE 8.—Structural formula for adduct II.

TABLE 5.—*The 270 and 500 MHz ^{1}H-NMR data for 2-NA adduct II* [a]

Chemical shift, ppm from TMS	Multiplicity (No. of protons)	Assignments
9.22[b]	S (1)	N^6-H (adenine)
8.46[c]	S (1)	H-2 or 8 (adenine)
8.05	S (1)	H-8 or 2 (adenine)
7.70	D[d] (1)	H-4,5, or 8 (naphthyl)
7.64	D[e] (1)	H-8,5, or 4 (naphthyl)
7.43	D[e] (1)	H-5,8, or 4 (naphthyl)
7.24	T (1)	H-7 (naphthyl)
7.00	T }	H-6 (naphthyl)
6.99	D[f] (2)	H-3 (naphthyl)
6.34	M (1)	H-1′ (dR)
5.17[b]	S (2)	N^2 -H_2 (naphthyl)
4.40	M (1)	H-3′ (dR)
3.90	M (1)	H-4′ (dR)
3.1–3.7	—	H_2O
2.75	M (1)	H-2′ or 2″ (dR)
2.51	—	d_5-DMSO
2.31	M (1)	H-2″ or 2′ (dR)

[a] We dissolved 50- to 200-μg samples in 0.1 ml d_6-DMSO. The following protons were unresolved: OH-3′ (dR) and OH-5′ (dR) due to line broadening; and H-5′ (dR) and H-5″ (dR) due to H_2O overlap. M = multiplet; T = triplet; D = doublet; S = singlet. The solvent was d_6-DMSO.

[b] This singlet is exchangeable with D_2O.

[c] This is a broad singlet that sharpens upon addition of NaOD (0.1 M).

[d] J_{ortho} = 8 Hz.

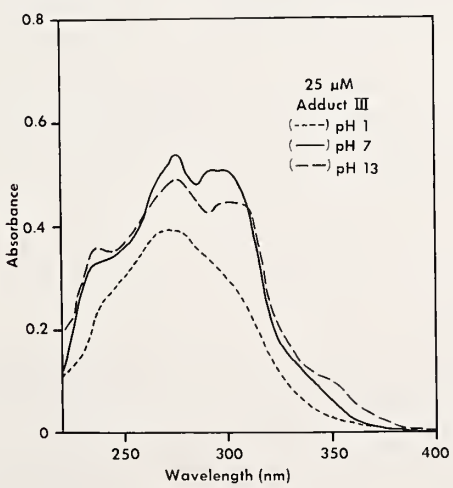
[e] J_{ortho} = 9 Hz.

[f] J was not determined due to resonance overlap.

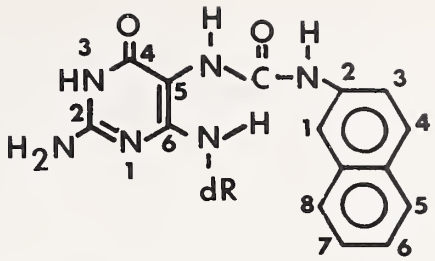
perimental conditions. The exchangeable singlet at 9.22 ppm with an intensity of one proton was assigned to N^6-H, indicating this position as the site of base substitution. Taken together, these results strongly support characterization of adduct II as 1-(dA-N^6-yl)-2-NA.

Identification of Adduct III as a Derivative of *N*-(Deoxyguanosin-8-yl)-2-naphthylamine

The major DNA adduct formed from *N*-OH-2-NA was analyzed by both high-resolution electron impact and low-resolution field desorption mass spectrometry (table 2). These data were consistent with a deoxyguanosinylnaphthylamine derivative with 5 sites available for silylation and thus suggested an *N*-(dG-8-yl)-2-NA structure. This conclusion was consistent with the pH-partitioning characteristics (text-fig. 3) which showed the presence of ionizable groups at both acid and alkaline pH. Furthermore, the UV spectra of adduct III (text-fig. 9) at pH's 1, 7, and 13 were vastly different from that observed for adducts I and II (text figs. 5, 7; table 3) with a strong diminution of the absorbance at 240 and 320 nm due to the naphthyl group. However, NMR spectroscopic analysis indicated some discrepancies with this structure (text-fig. 10, table 6). The presence of five exchangeable resonances in the downfield region was incompatible with an intact C-8-substituted dG adduct. Yet all 7 nonexchangeable naphthyl protons were still present, and the *ortho* H-1 and H-3 protons had apparently shifted downfield by nearly 1 ppm, a property observed for other C-8-guanine–arylamine adducts [(*7, 31*); Kadlubar et al: Unpublished data]. Therefore, we suggest that after its formation or during these isolation procedures, *N*-(dG-8-yl)-2-NA is hydrolyzed across its (8–9)-guanine bond to yield 1-[5-(2,6-diamino-4-oxopyrimidinyl-N^6-deoxyriboside)]-2-(2-naphthyl)urea. With mass spectral analyses under high vacuum and elevated temperatures or during sample preparation, the adduct may dehydrate and close the guanine ring. However, additional mass and NMR spectral studies will be required to resolve these differences.



TEXT-FIGURE 9.—UV spectra of adduct III in 0.1 N HCl (- - -), 10 mM potassium citrate at pH 7 (——), and 0.1 N NaOH (– – –).



TEXT-FIGURE 10.—Structural formula for adduct III.

TABLE 6.—*The 270 MHz ^{1}H-NMR data for 2-NA adduct III* [a]

Chemical shift, ppm from TMS	Multiplicity (No. of protons)	Tentative assignments
10.8[b]	S (1)	*N*-3-H (pyrimidine)
8.85[b]	S (1)	N^2-H (naphthyl) or N^5-H (pyrimidine)
8.55[b]	S (1)	N^5-H (pyrimidine) or N^2-H (naphthyl)
8.36	S (1)	H-1 (naphthyl)
7.7–7.8	M (4)	H-3, 4, 5 and 8 (naphthyl)
7.44	T (1)	H-7 (naphthyl)
7.32	T (1)	H-6 (naphthyl)
6.49[b]	S (2)	N^2-H_2 (pyrimidine)
6.37	M (1)	H-1′ (dR)
6.03[b, c]	M (1)	OH-5′ (dR)
5.35[b, c]	M (2)	N^6-H (pyrimidine) and OH-3′ (dR)
4.45	S (1)	H-3′ (dR)
3.96	S (1)	H-4′ (dR)
3.80	S (2)	H-5′ and 5″ (dR)
3.0–3.6	—	H_2O
2.51	—	d_5-DMSO
2.05	M (2)	H-2′ and 2″ (dR)

[a] We dissolved 100- to 500-µg samples in 0.25 ml d_6-DMSO. M = multiplet; T = triplet; D = doublet; S = singlet.

[b] This peak was exchangeable with D_2O.

[c] The assignment of these broad peaks are uncertain because this region contained several low intensity peaks due to partial sample decomposition.

Nevertheless, a ring-opened, C-8-substituted dG structure is indicated from studies on the relative stability of adducts I, II, and III at pH 1, 7, and 13 (table 7; *see* "Materials and Methods"). In contrast to adducts I and II which appear to be readily depurinated under acidic conditions, adduct III is acid stable. Inasmuch as other C-8-substituted arylamine derivatives also depurinate under acidic conditions (*6, 29, 31–33*), a ring-opened structure for adduct III seems plausible. Its base lability may be due to the presence of a "substituted-urea" functional group at the (pyrimidine)N^5H-(C=O)-NH(naphthyl) linkage.

Preliminary Experiments on the Macromolecular Binding of [^{3}H]2-Naphthylamine In Vivo

To determine whether or not any of the DNA adducts formed in vitro by reaction with *N*-OH-2-NA are also formed in vivo, we administered [^{3}H]2-NA (100 mCi/

TABLE 7.—*Relative stabilities of adducts I, II, and III at pH 1, 7, and 13*

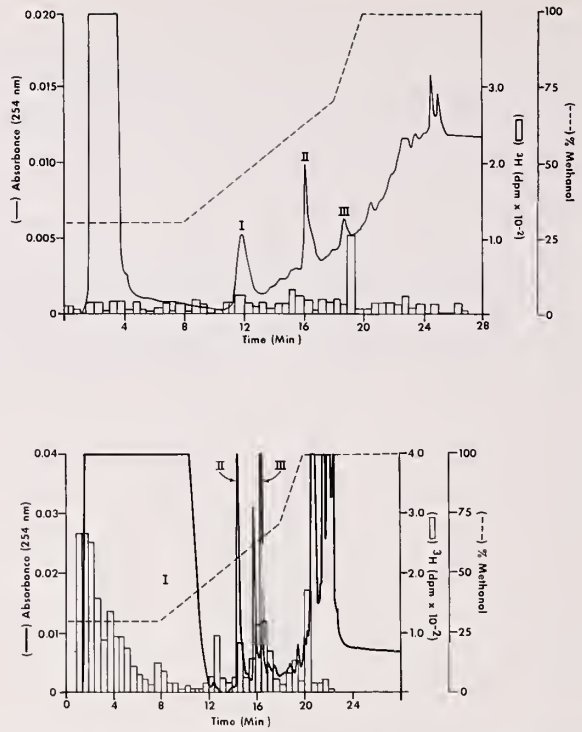
Adducts	Percent recovery (37° C/4 hr) at:		
	pH 1	pH 7	pH 13
I	10[a]	95	95
II	20[a]	95	95
III	95	95	<5[b, c]

[a] Adduct is converted to a more nonpolar product.
[b] Adduct is decomposed to several products.
[c] Less than 5% is judged the limit of detection.

TABLE 8.—*In vivo binding of [^{3}H]2-NA to cellular macromolecules*[a]

Tissue	pmol [^{3}H]2-NA bound/mg:		
	DNA	RNA	Protein
Bladder epithelium	1.7	14.0	20.8
Liver	3.6	9.5	13.0

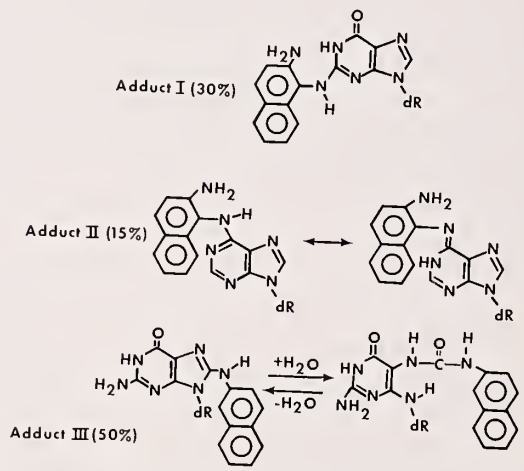
[a] [^{3}H]2-NA (100 mCi/mmol) was administered orally to an adult male beagle at a dose of 8 mg/kg body wt. After 24 hr, the dog was killed, and tissue macromolecules were isolated (*see* "Materials and Methods").



TEXT-FIGURE 11.—HPLC radiochromatograms of enzymatic hydrolysates of bladder epithelial DNA (*upper frame*) and hepatic DNA (*lower frame*) from a dog given [^{3}H]2-NA (100 mCi/mmol. The position of the adducts added as UV markers and the solvent composition are indicated on the figure; the flow rate was 2 ml/min.

mmol) orally to an adult male beagle at a dose of 8 mg/kg. After 24 hours, the dog was killed, and DNA, RNA, and protein were isolated from the bladder epithelium, a target tissue for 2-NA carcinogenesis, and from the liver, a non-target tissue. As shown in table 8, covalent binding of 2-NA was detected in both tissues (protein> RNA> DNA). Similar levels of binding of 2-NA to mouse (*34*) and rat (*35*) hepatic macromolecules have been reported.

The dog urothelial and hepatic DNA's were enzymatically hydrolyzed and analyzed by HPLC after addition of unlabeled adducts I, II, and III as UV markers. These data, although preliminary, indicate the presence of the C-8 dG-(2-NA) derivative (adduct III) in urothelial DNA (text-fig. 11, top). In the hepatic DNA hydrolysate, a complex radiochromatogram was obtained (text-fig. 11, bottom). Although most of the ^{3}H migrated with early eluting components, small amounts of the radioactivity were associated with adducts II and III, and possibly adduct I; the marker was obscured by UV-absorbing components in the extract. Additional studies are in progress in which highly tritiated 2-NA (1Ci/mmol) is used so that the presence, persistence, and repair of these and other adducts can be accurately determined and related to 2-NA-induced urinary bladder carcinogenesis.



TEXT-FIGURE 12.—Structure of (2-NA)-DNA adducts I, II, and III.

DISCUSSION

In contrast to *N*-OH-1-NA which reacts with DNA at the O^6 position of the guanine base, *N*-OH-2-NA reacts at exocyclic amino groups of guanine and adenine and at the C-8 position of guanine (text-fig. 12). The reaction at the N^2 of guanine, which accounts for 30% of the *N*-OH-2-NA bound to DNA in vitro, has also been observed by the reaction of nucleic acids with ultimate carcinogenic derivatives of several aromatic amines (*29, 30, 36*) and polycyclic hydrocarbons (*37–40*). Due to their persistence in target tissues or to their formation as major adducts of strong carcinogens, N^2-guanine adducts have been suggested to play a central role in the initiation of neoplasia. Although it is not apparent how the N^2 adducts may induce a herit-

able lesion, the N^6-adenine adducts, which are DNA products derived from certain aromatic amines (*33, 41*) and benzo[*a*]pyrene (*39*) and account for 15% of *N*-OH-2-NA bound to DNA in vitro, may constitute promutagenic, mispairing lesions by virtue of their possible tautomeric equilibria between N^6-amino and -imino forms (text-fig. 12). In the latter form, mispairing with deoxycytidine might occur and result in a transition mutation. The C-8 dG-*N*-OH-2-NA adduct, which accounts for 50% of the *N*-OH-2-NA bound to DNA in vitro, may be present in cellular DNA in either intact and/or ring-opened forms (text-fig. 12). Studies with space-filling molecular models (Cory-Pauling-Koltun Precision Molecular Models) obtained from the Ealing Corporation (South Natick, Mass.) of DNA containing bound 2-NA indicate that, unlike adducts I and II, adduct III in its opened form would cause considerable local distortion and destabilization of the double helix and may thus serve as a stimulus for DNA replication. This could then promote the fixing of a mispairing lesion such as adduct II or the misrepair of adduct I.

If reaction with DNA is a critical event in *N*-OH arylamine carcinogenesis, then it becomes necessary to explain the following observations: *N*-OH-1-NA reacts with DNA in vitro tenfold more than does *N*-OH-2-NA (*9*), and it is considerably more carcinogenic at subcutaneous sites of application (Miller EC, Kadlubar FF, Scribner JD, et al: Unpublished data). Yet the relative carcinogenicities of 1- and 2-NA for the dog urinary bladder are the opposite (*19*), although both *N*-OH-1-NA and 2-NA are formed as urinary metabolites (*19, 21*). From our studies on the nature of the DNA adducts formed from *N*-OH-1-NA and -2-NA, we offer the following rationalizations: 1) In the urinary bladder, the presumed reaction of DNA with *N*-OH-1-NA at O^6 of guanine would not be expected to cause major structural perturbations in the helix and induce DNA synthesis; thus the lesion may not be sufficient to fix a heritable change that leads to neoplasia. 2) Alternatively, the reaction of *N*-OH-2-NA with DNA, although at a lower level of modification, may induce lesions which may not only cause mispairing or misrepair but may also fix the damage through induction of DNA synthesis. Therefore, in the urothelium, the N-OH metabolite of 1-NA may be an incomplete carcinogen, whereas *N*-OH-2-NA would be effective as a complete ultimate carcinogen.

However, at subcutaneous sites of application, *N*-OH-1-NA may exhibit its greater carcinogenicity due to its greater reaction with DNA. The local stress and resultant hyperplasia associated with repeated sc injections could stimulate DNA synthesis and fix the damage.

Our carcinogenicity studies currently in progress will assess the synergistic, co-carcinogenic, or tumor initiating and promoting properties of *N*-OH-1-NA and -2-NA. These studies may be particularly relevant to the human situation in which simultaneous exposure to both 1- and 2-NA occurred (*17, 19*).

REFERENCES

(*1*) Yugawa T: Studies on the synthesis of *p*-phenetidine and its reaction mechanism. 2. Reaction mechanism for synthesis of *p*-phenetidine. Nippon Kagaku Zasshi 71: 603–605, 1950

(*2*) Heller HE, Hughes ED, Ingold CK: A new view of arylhydroxylamine rearrangement. Nature 168:909–910, 1951

(*3*) Boyland E, Manson D, Nery R: The reaction of phenylhydroxylamine and 2-naphythylhydroxylamine with thiols. J Chem Soc (Lond):606–611, 1962

(*4*) Kriek E: On the interaction of *N*-fluorenylhydroxylamine with nucleic acids in vitro. Biochem Biophys Res Commun 20:793–799, 1965

(*5*) _____: Reactions of *N*-2-fluorenylhydroxylamine with nucleic acids in vitro. *In* Carcinogenesis, A Broad Critique (M.D. Anderson and Tumor Institute). Baltimore: Williams & Wilkins, 1967, pp 441–446

(*6*) King CM, Phillips B: *N*-Hydroxy-2-fluorenylacetamide. Reaction of the carcinogen with guanosine, ribonucleic acid, deoxyribonucleic acid, and protein following enzymatic deacetylation or esterification. J Biol Chem 244:6209–6216, 1969

(*7*) Beland FA, Allaben WT, Evans FE: Acyltransferase mediated binding of N-hydroxyarylamides to nucleic acids. Cancer Res 40:834–840, 1980

(*8*) Beland FA, Dooley KL, Evans FE, et al: Role of persistent DNA-bound residues of *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) in tumor induction. Proc Am Assoc Cancer Res 20:518, 1979

(*9*) Kadlubar FF, Miller JA, Miller EC: Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res 37:805–814, 1977

(*10*) Radomski JL, Hearn WL, Radomski T, et al: Isolation of the glucuronic acid conjugate of *N*-hydroxy-4-aminobiphenyl from dog urine and its mutagenic activity. Cancer Res 37:1757–1762, 1977

(*11*) Kadlubar F, Flammang T, Unruh L: The role of N-hydroxy arylamine and N-glucuronides in arylamine-induced urinary bladder carcinogenesis: Metabolite profiles in acidic, neutral and alkaline urines of 2-naphthylamine- and 2-nitronaphthalene-treated rats. *In*: Conjugation Reactions in Drug Biotransformation (Aitio A, ed). Amsterdam: Elsevier/North Holland, 1978, pp 443–454

(*12*) Kadlubar FF, Miller JA, Miller EC: Guanyl O^6-arylamination and O^6-arylation of DNA by the carcinogen N-hydroxy-1-naphthylamine. Cancer Res 38:3628–3638, 1978

(*13*) Loveless A: Possible relevance of O^6-alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature 223:206–208, 1969

(*14*) Lawley PD: Carcinogenesis by alkylating agents. *In:* Chemical Carcinogens, American Chemical Society Monogr No. 173 (Searle CE, ed). Washington, D.C.: Am Chem Soc, 1976, pp 83–244

(*15*) Belman S, Troll W, Teebor G, et al: The carcinogenic and mutagenic properties of N-hydroxy-aminonaphthalenes. Cancer Res 28:535–542, 1968

(*16*) Radomski JL, Brill E, Deichmann WB, et al: Carcinogenicity testing of N-hydroxy and other oxidation and decomposition products of 1- and 2-naphthylamine. Cancer Res 31:1461–1467, 1971

(*17*) Case RA, Hosker ME, McDonald DB, et al: Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Br J Ind Med 11:75–104, 1954

(*18*) Price JM: Etiology of bladder cancer. *In:* Benign and

Malignant Tumors of the Urinary Bladder (MALTRY E JR, ed). Flushing, N.Y.: Medical Examination, 1971, pp 189–261

(*19*) RADOMSKI JL: The primary aromatic amines: Their biological properties and structure–activity relationships. Annu Rev Pharmacol Toxicol 19:129–157, 1979

(*20*) RADOMSKI JL, BRILL E: Bladder cancer induction by aromatic amines: Role of N-hydroxy metabolites. Science 167:992–993, 1970

(*21*) _____: The role of N-oxidation products of aromatic amines in the induction of bladder cancer in the dog. Arch Toxicol 28:159–175, 1971

(*22*) HEARN WL, RADOMSKI JL: Rearrangement of 1-naphthylhydroxylamine (1-NOH) and its N-glucuronide under mildly acidic conditions as it relates to bladder cancer. Proc Am Assoc Cancer Res 20:472, 1979

(*23*) BOYLAND E, DUKES CE, GROVER PL: Carcinogenicity of 2-naphthylhydroxylamine and 2-naphthylamine. Br J Cancer 17:79–84, 1963

(*24*) BELAND FA, DOOLEY KL, CASCIANO DA: Rapid isolation of carcinogen-bound DNA and RNA by hydroxyapatite chromatography. J Chromatogr 174:177–176, 1979

(*25*) KADLUBAR FF, MILLER JA, MILLER EC: Microsomal N-oxidation of the hepatocarcinogen N-methyl-4-aminoazobenzene and the reactivity of N-hydroxy-*N*-methyl-4-amino-azobenzene. Cancer Res 36:1196–1206, 1976

(*26*) MANSON D: Oxidation of naphthylhydroxylamines to nitrosonaphthols by air. J Chem Soc [Perkin I]:192–194, 1974

(*27*) MOORE PD, KOREEDA M: Application of the change in partition coefficients with pH to the structure determination of alkyl-substituted guanosines. Biochem Biophys Res Commun 73:459–464, 1976

(*28*) GATLIN L, DAVIS JC JR: Comparison of ribose and deoxyribose nucleosides by N.M.R. and deductions regarding ribose and deoxyribose nucleic acids. 1. Tautomeric forms. J Am Chem Soc 84:4464–4470, 1962

(*29*) BELAND FA, TULLIS DL, KADLUBAR FF, et al: Identification of the DNA adducts formed in vitro from *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene and in rat liver in vivo after administration of *N*-methyl-4-aminoazobenzene. Natl Cancer Inst Monogr 58:153–161, 1981

(*30*) WESTRA JG, KRIEK E, HITTENHAUSEN H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149–164, 1976

(*31*) LIN J-K, SCHMALL B, SHARPE ID, et al: N-Substitution of carbon-8 in guanosine and deoxyguanosine by the carcinogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene in vitro. Cancer Res 35:832–843, 1975

(*32*) KRIEK E, MILLER JA, JUHL U, et al: 8-(*N*-2-Fluorenylacetamido)guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide in neutral solution. Biochemistry 6:177–182, 1967

(*33*) SCRIBNER JD, NAIMY NK: Adducts between the carcinogen 2-acetamidophenanthrene and adenine and guanine in DNA. Cancer Res 35:1416–1421, 1975

(*34*) HUGHES PE, PILCZYK R: The in vivo binding of metabolites of 2-naphthylamine to mouse liver DNA, RNA, and protein. Chem Biol Interact 1:307–314, 1969/70

(*35*) ROBERTS JJ, WARWICK GP: The covalent binding of metabolites of dimethylaminoazobenzene, β-naphthylamine, and aniline to nucleic acids in vivo. Int J Cancer 1:179–196, 1966

(*36*) KRIEK E, HENGEVELD GM: Reaction products of the carcinogen *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl with DNA in liver and kidney of the rat. Chem Biol Interact 21:179–201, 1978

(*37*) DIPPLE A, BROOKES P, MACKINTOSH DS, et al: Reaction of 7-bromo-methylbenz[*a*]anthracene with nucleic acids, polynucleotides, and nucleosides. Biochemistry 10: 4323–4330, 1971

(*38*) JEFFREY AM, JENNETTE KW, BLOBSTEIN SH, et al: Benzo[*a*]pyrene-nucleic acid derivative found in vivo: Structure of a benzo[*a*]pyrenetetrahydrodiol epoxide–guanosine adduct. J Am Chem Soc 98:5714–5715, 1976

(*39*) STRAUB KM, MEEHAM T, BURLINGAME A, et al: Identification of the major adducts formed by reaction of benzo[a]-pyrene diol epoxide with DNA in vitro. Proc Natl Acad Sci USA 74:5285–5289, 1977

(*40*) JEFFREY AM, BLOBSTEIN SH, WEINSTEIN IB, et al: Structure of 7,12-dimethylbenz[*a*]anthracene–guanosine adducts. Proc Natl Acad Sci USA 73:2311–2315, 1976

(*41*) KAWAZOE Y, ARAKI M, HUANG E-F, et al: Chemical structure of QA_{II}, one of the covalently bound adducts of carcinogen 4-nitroquinoline 1-oxide with nucleic acid bases of cellular nucleic acids. Chem Pharm Bull 23:3041–3043, 1975

Identification of the DNA Adducts Formed In Vitro From *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene and in Rat Liver In Vivo After Administration of *N*-Methyl-4-aminoazobenzene[1]

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ABSTRACT—The synthetic model ultimate carcinogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene reacted in vitro with either calf thymus or rat liver DNA to yield approximately 1 bound residue per 1,000 nucleotides. The DNA was enzymatically hydroyzed and subjected to high-pressure liquid chromatographic analysis which indicated the presence of at least 6 *N*-methyl-4-aminoazobenzene (MAB) adducts. Two of the products cochromatographed with MAB–DNA adducts formed in rat liver in vivo following oral administration of the precarcinogen MAB. These two adducts were identified by UV, mass, and nuclear magnetic resonance spectroscopy as *N*-(deoxyguanosin-8-yl)- and 3-(deoxyguanosin-N^2-yl)-MAB; the first adduct was initially the predominant product in vivo, but it could not be detected 7 days after treatment, and the second remained at a constant level for 14 days and therefore appeared to be a persistent lesion.—Natl Cancer Inst Monogr 58: 153–161, 1981.

In 1937, Kinosita et al. (*1*) demonstrated the amino azo dye DAB (butter yellow) to be a liver carcinogen. Inasmuch as this was the first instance in which a carcinogen induced tumors at a location distant from the site of application, their discovery suggested that metabolism was required before the azo dye could exert its oncogenic effect. Therefore, during the succeeding four decades, this compound and a number of its analogs have been intensely studied so that the precise steps involved in tumor induction by amino azo dyes could be defined. Although many questions remain unanswered, a much clearer picture now exists of the metabolic pathways through which this class of compounds is activated. The first discovery, centered around the importance of the azo linkage, was a result of the inability of American researchers to repeat the findings of Kinosita. The discrepancy was traced to differences in the diet; in the Kinosita study, a riboflavin-deficient, polished rice diet was used. Subsequent studies indicated that an intact azo linkage was required for these compounds to be carcinogenic (*2–4*) and that the enzyme system responsible for azo bond reduction required riboflavin as a cofactor (*5*). Thus in the original tumor experiment, this metabolic pathway was fortuitously and unknowingly inhibited. More direct evidence that azo reduction was a deactivation step came from the use of the end products of this metabolic sequence in tumor induction experiments. When the azo linkage of DAB is cleaved, aniline and *N,N*-dimethyl-4-phenylenediamine are formed; these compounds failed to elicit tumors when fed to rats at levels comparable to DAB (*2–4*).

The oxidative N-demethylation of DAB to MAB is catalyzed by the cytochrome P_{450} enzyme system and has an absolute requirement for NADPH and oxygen (*6*). In contrast to azo reduction, this N-demethylation step is regarded as a metabolic activation step, inasmuch as MAB was as potent as DAB in eliciting liver tumors in rats (*7*). However, subsequent N-demethylation of MAB to AB represents a deactivation step because this compound has been, at best, a weak hepatocarcinogen in rats (*7*). Apparently, an intact azo linkage and an N-methyl group are two structural requirements necessary for appreciable tumorigenic activity in this species. Recent data indicate that AB is a potent carcinogen in the newborn mouse (*8*).

Following metabolic N-demethylation, the amino azo dye can become N-acetylated. This sequence readily occurs as demonstrated by the fact that after dietary administration of DAB, MAB, or AB, *N*-acetyl-AB was detected as a urinary metabolite. Although other aromatic amides, notably *N*-2-fluorenylacetamide, are potent hepatocarcinogens, *N*-acetyl-AB failed to elicit tumors when administered to rats (*9*). The same was true with the presumably more potent derivatives, *N*-OH-*N*-acetyl-AB and *N*-acetoxy-*N*-acetyl-AB (*9*). Thus N-acetylation must be regarded as a deactivation process in the metabolism of amino azo dyes.

Abbreviations: DAB = *N,N*-dimethyl-4-aminoazobenzene; MAB = *N*-methyl-4-aminoazobenzene; AB = 4-aminoazobenzene; OH = hydroxy; *N*-BzO-MAB = *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene; NMR = nuclear magnetic resonance; Bis-Tris = bis(2-hydroxyethyl)-tris(hydroxymethyl)-aminomethane base; HPLC = high-pressure liquid chromatography; d_6, d_5 = deuterated; DMSO = dimethyl sulfoxide; TMS = tetramethylsilane; ppm = parts per million; m/z = mass divided by elementary charge; BSTFA = bis(trimethylsilyl)-trifluoroacetamide; dG = deoxyguanosin; dR = deoxyribose.

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Ring-hydroxylation is a common metabolic pathway for aromatic amines. These phenolic metabolites may arise through either of two pathways: 1) epoxidation followed by isomerization, or 2) N-hydroxylation followed by rearrangement of either the hydroxylamine or its O-esters to phenolic amides. Whereas the phenolic metabolites of DAB and MAB have been demonstrated to be noncarcinogenic (*7, 10*), the epoxide intermediates, at least in theory, could be ultimate carcinogenic species. Available evidence argues against this, however. A major phenolic metabolite of DAB is 4′-OH-DAB which could arise from 3′,4′-epoxide. If this arene oxide was responsible for the tumorigenic action of DAB, then inhibition of epoxidation at these positions should abolish tumor formation. Methyl substitution generally blocks (*11*) or at least retards epoxidation (*12*), and yet both 3′- and 4′-methyl-DAB are potent hepatocarcinogens (*7*), a finding that implies that arene oxides do not play a significant role in DAB- and MAB-induced tumors.

As mentioned previously, an N-methyl group is required for appreciable tumorigenic activity in the rat. For MAB, this amine function is oxidized by the microsomal flavoprotein mixed function oxidase (*13*), which requires NADPH and molecular oxygen. The product of this reaction is *N*-OH-MAB, an unstable compound which can undergo further oxidation to a nitrone. This latter product in turn hydrolyzes to give *N*-OH-AB and formaldehyde (*13*). Both *N*-OH-MAB and -AB readily react with protein, presumably through their respective nitrone and nitroso derivatives (*13*). However, Kadlubar et al. (*13, 14*) demonstrated that metabolic conversion of *N*-OH-MAB to a reactive ester must take place for nucleic acid binding to occur. Labuc and Blunck (*15*), using the potent hepatocarcinogen 3′-methyl-DAB, reached these same conclusions. They observed that although protein binding could occur in the presence of only microsomes and NADPH, the addition of cytosol and 3′phosphoadenosine-5′phosphosulfate was necessary to obtain nucleic acid binding.

In principle, *N*-OH-MAB could be converted into a reactive ester by several systems. The following activation pathways have been investigated: acetyl-CoA-dependent O-acetylation, ATP-dependent O-serylation, *S*-adenosylmethionine-dependent O-methylation, or 3′ phosphoadenosine-5′-phosphosulfate-dependent O-sulfonation. However, only through the last pathway could a time-dependent loss of *N*-OH-MAB be demonstrated (*14*). This result and the observation that the addition of sodium sulfate to the diet increased the carcinogenicity of 3′-methyl-DAB (*16*) suggest that MAB-*N*-sulfate may be the sole ultimate carcinogenic form of DAB and MAB.

A dominant theme in chemical carcinogenesis is that the interaction of ultimate carcinogens with informational macromolecules, notably DNA, represents an initiation event. Thus the addition of sulfate to a diet containing MAB could cause an increased tumor incidence either by elevating the level of MAB–DNA adducts or by altering the variety of adducts. One of our long-range goals here is to investigate this question. However, at the onset, we believed it necessary to determine whether multiple MAB–DNA adducts are formed in vivo. Although one product has previously been identified as *N*-(dG-8-yl)-MAB (*17*), we thought additional adducts should be formed based on comparisons with other DNA-reactive carcinogens. Therefore, we reinvestigated the identity of the MAB–DNA adduct(s) that are formed in rat liver in vivo and conducted reactions between the ultimate carcinogen *N*-BzO-MAB and DNA. The adducts obtained were compared with those formed in vivo after MAB was administered. When adducts common to both systems were found, the in vitro products were identified by spectroscopic techniques and the in vivo persistence of individual adducts was determined.

MATERIALS AND METHODS

Chemicals.—We synthesized (prime ring-^{3}H)-MAB (76 mCi/mmol) according to the method of Lin and Miller (*18*) and (prime-ring-^{3}H)-*N*-BzO-MAB (70 mCi/mmol) as described in (*13*). The following distribution of the ^{3}H label was established with the use of ^{3}H-NMR spectroscopy: Seventy percent of the label is located at positions 2′ and 6′ and 30% at the 4′ position. The *N*-BzO-MAB was obtained from Drs. William Duncan and Robert Roth of the Midwest Research Institute (Kansas City, Mo.) and DNase I (DN-Cl), snake venom phosphodiesterase (type VII), alkaline phosphatase (type III-S), trioctanoin, and calf thymus DNA (highly polymerized, type I) were purchased from the Sigma Chemical Company (St. Louis, Mo). (All other chemicals were reagent grade or better and were purchased from common laboratory supply houses.) Rat liver DNA, which was used in the in vitro binding studies, was isolated by hydroxyapatite chromatography (*19*).

Instrumentation.—The UV spectra were recorded on a Cary model 219 (Palo Alto, Calif.); ^{1}H-NMR spectra were obtained with a WH 270 spectrometer from Bruker Instruments, Inc. (Billerica, Mass.) that was operated in the Fourier transform mode. Field-desorption and high-resolution electron impact mass spectra were acquired with a Kratos-AEI (Manchester, U.K.) MS 902. Radioactivity was measured in Scintisol purchased from Isolab Inc. (Akron, Ohio) with a Mark III liquid scintillation spectrometer obtained from G. D. Searle & Co. (Des Plaines, Ill.). We performed HPLC on a Waters Associates, Inc. (Milford, Mass.) system, which consisted of 2 model 6000A pumps, a model 660 solvent programmer, a U6K injector, and a model 440 UV detector.

In vitro DNA binding.—Fifty milligrams calf thymus or rat liver DNA was dissolved in 10 ml 5 mM Bis-Tris–HCl buffer, pH 7.1. This solution was purged with argon and then treated with 3.3 mg *N*-BzO-MAB (0.01 mmol) which had previously been dissolved in 200 μl of argon-purged 95% ethanol. The combined mixture was incubated at 37° C for 1 hour and then the MAB–DNA was isolated as described in (*13, 20*).

The DNA (1 mg/ml) was hydrolyzed in 5 mM Bis-Tris–10 mM magnesium chloride buffer, pH 7.1. We added 0.1 mg DNase I/mg DNA, and after a 6-hour incubation at 37° C, the pH was adjusted to 9.0 with 1 M Tris-base. One unit of snake venom phosphodiesterase/25 mg DNA and 1 U of alkaline phosphatase/mg DNA were then added, and the incubation was resumed for 18 hours.

The MAB–DNA adducts were isolated by extraction of the hydrolysate twice with equal volumes of water-saturated, redistilled *n*-butanol. These organic layers were combined, washed once with water, and evaporated under reduced pressure; then the residue was dissolved in a minimal volume of methanol for analysis by HPLC. We separated the individual adducts on a μBondapak-C^{18} column obtained from Waters Associates, Inc. by running a 30-minute convex gradient (Waters curve 2) from 20 to 60% methanol with a 2-ml/minute flow rate.

In vivo DNA binding.—Male 150-g Sprague-Dawley rats from our breeding colony were fed a low riboflavin (1 mg riboflavin/kg feed) diet for 7 days preceding MAB administration. They were then given orally 0.2 mmol (prime ring-^{3}H)-MAB/kg dissolved in trioctanoin (0.4 ml of 0.05 M MAB in trioctanoin). At approximately 8 hours or 1, 7, or 14 days after treatment, the animals were decapitated. The livers were excised, and DNA was obtained through the use of hydroxyapatite chromatography as in (*19*). To have sufficient radioactivity for adduct analysis, we combined the DNA from three livers. This fraction was enzymatically hydrolyzed to mononucleosides, and the adducts were separated by the procedures outlined above.

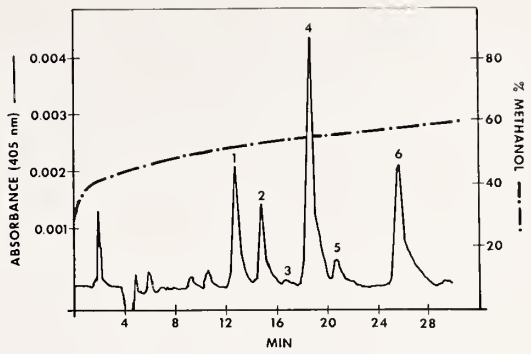
Miscellaneous.—The pH-dependent partitioning experiments were conducted according to the method of Moore and Koreeda (*21*) with (prime ring-^{3}H)-*N*-BzO-MAB. The NMR spectra were obtained in 99.96% DMSO-d_6. We referenced the chemical shifts to tetramethylsilane by defining the DMSO-d_5 resonance as 2.51 ppm. Because MAB–DNA adducts were expected to be photolabile, we performed these experiments under yellow, low-UV fluorescent lights produced by General Electric Company (gold, No. 4060; Cleveland, Ohio).

RESULTS

In Vitro Binding of *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene to DNA

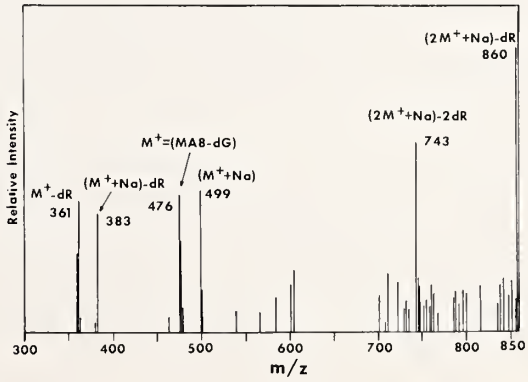
Investigators have used *N*-BzO-MAB as a synthetic analog for the in vivo ultimate carcinogenic ester(s) of *N*-OH-MAB. This synthetic ester reacts readily with cellular macromolecules (*22*) and has induced sarcomas directly at application sites (*8, 22, 23*). These results suggested that the DNA adducts formed from *N*-BzO-MAB may be representative of those formed in vivo from the metabolically generated ester(s) of *N*-OH-MAB. In addition, *N*-BzO-MAB can be chemically synthesized and reacted with DNA in vitro to yield sufficient quantities of adducts with structures that can be unambigously determined.

When *N*-BzO-MAB was reacted with either rat liver or calf thymus DNA it yielded approximately 1 MAB residue/1,000 nucleotides. The DNA was enzymatically hydrolyzed, and the adducts were partitioned into *n*-butanol (*see* "Materials and Methods"). Our HPLC analysis of the *n*-butanol extract indicated that at least 6 adducts were present (text-fig. 1). Two of the products, peaks 2 and 4, cochromatographed with MAB-DNA adducts obtained in vivo and were isolated and subjected to extensive characterization.



TEXT-FIGURE 1.—HPLC profile of MAB–DNA adducts obtained when *N*-BzO-MAB reacted with rat liver DNA. Similar results were obtained when *N*-BzO-MAB reacted with calf thymus DNA. Adducts II and IV cochromatographed with MAB adducts found in vivo.

Field desorption mass spectroscopy of adduct IV revealed predominant peaks at *m/z* 499, 476, 383, and 361 (text-fig. 2). These peaks, which were consistent with a deoxyguanosinyl–MAB adduct, represented deoxyguanosinyl–MAB plus sodium, deoxyguanosinyl–MAB, guaninyl–MAB plus sodium, and guaninyl–MAB, respectively. Furthermore, the latter two fragments indicated that MAB substitution had to be through the purine moiety. Clustered ions observed at *m/z* 975, 860, and 743 also supported this assignment. A comparable spectrum could not be obtained with adduct II because of its instability during analysis. Therefore, to acquire mass spectral data, we silylated the adducts with BSTFA–pyridine (4:1) at 60° C for 1 hour. High-resolution, electron-impact mass spectroscopy on these derivatives indicated that both compounds were dG products (table 1; text-fig. 3). Adduct II had a predominant parent ion of *m/z* 692 consistent with tris-TMS-deoxyguanosinyl-MAB, whereas adduct IV with a parent ion of *m/z* 764 corresponds to a tetrakis-TMS derivative. These data indicated that with adduct II, a covalent bond must have formed through a potentially silylatable position, i.e., O^6, N-1, or N^2 of dG, whereas for adduct IV, these positions must be unsubstituted. With



TEXT-FIGURE 2.—Field-desorption mass spectrum of MAB-DNA adduct IV. Peaks at *m/z* 743, 859, and 860 represent clustered ions. In addition to these, an intense peak was observed at *m/z* 975 (2 M^+ + Na).

TABLE 1.—*High-resolution, mass spectral data for silylated MAB-DNA adducts II and IV*[a]

Adduct	*m/z*	Elemental formula [error in ppm]		Assignment
II	692.3121	$C_{23}H_{21}N_8O_4TMS_3$	[2.04]	M^+ = (dG-MAB-TMS_3)
	432.1812	$C_{18}H_{15}N_8O_1TMS_1$	[−7.08]	M^+ − (dR-TMS_2)
IV	764.3481	$C_{23}H_{20}N_8O_4TMS_4$	[−2.76]	M^+ = (dG-MAB-TMS_4)
	749.3323	$C_{22}H_{17}N_8O_4TMS_4$	[7.43]	$M^+ - CH_3$
	692.3168	$C_{23}H_{21}N_8O_4TMS_3$	[8.88]	M'^+ = (dG-MAB-TMS_3)
	576.2633	$C_{18}H_{13}N_8O_1TMS_3$	[0.03]	M''^+ = (MAB-guanine-TMS_3)
	504.2193	$C_{18}H_{14}N_8O_1TMS_2$	[−8.89]	M^+ − (dR-TMS_2)
	489.1969	$C_{17}H_{11}N_8O_1TMS_2$	[−6.96]	M^+ − (dR-TMS_2)-CH_3
	432.1836	$C_{18}H_{15}N_8O_1TMS_1$	[−1.59]	M'^+ − (dR-TMS_2)

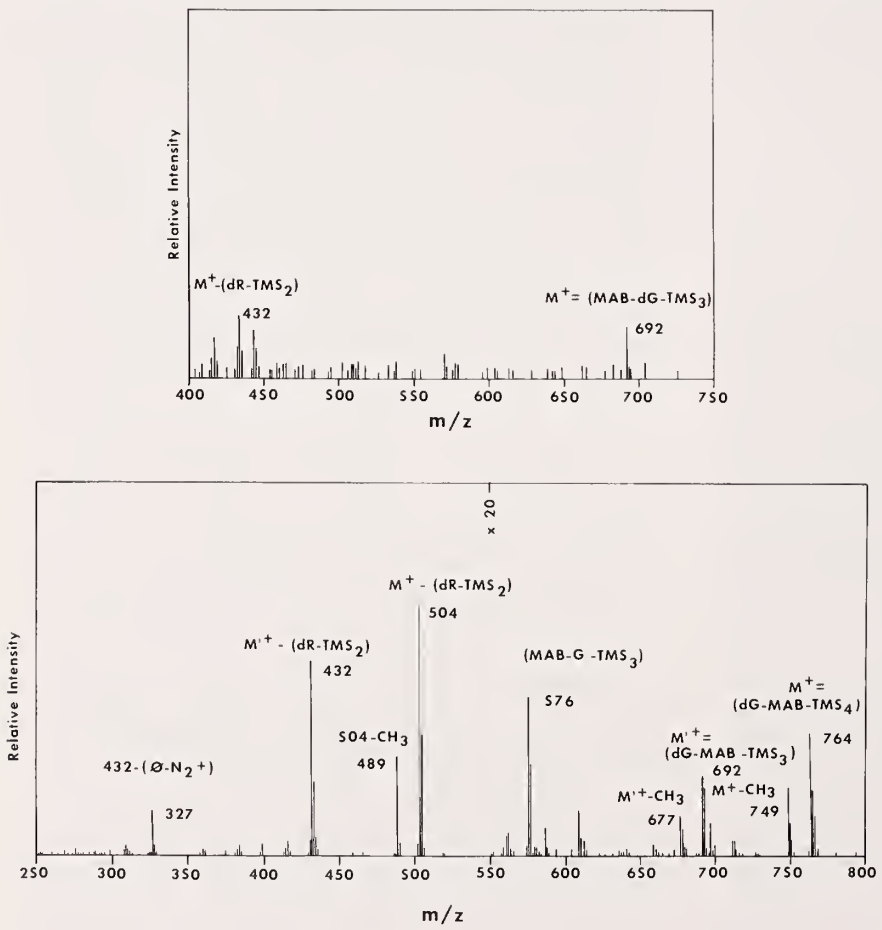
[a] Adducts were silylated with BSTFA–pyridine (4:1) at 60° C for 1 hr.

adduct II, a major fragment was observed at *m/z* 432 that corresponded to the loss of bis(trimethylsilyl)-deoxyribose. This finding suggested that MAB was covalently bound to the purine moiety. An analogous fragment was observed with silylated adduct IV (*m/z* 504), which confirmed the field desorption data. Additionally, the presence of a fragment at *m/z* 327 for adduct IV indicated loss of a phenyldiazonium ion that implied the substitution could not be through the MAB prime ring.

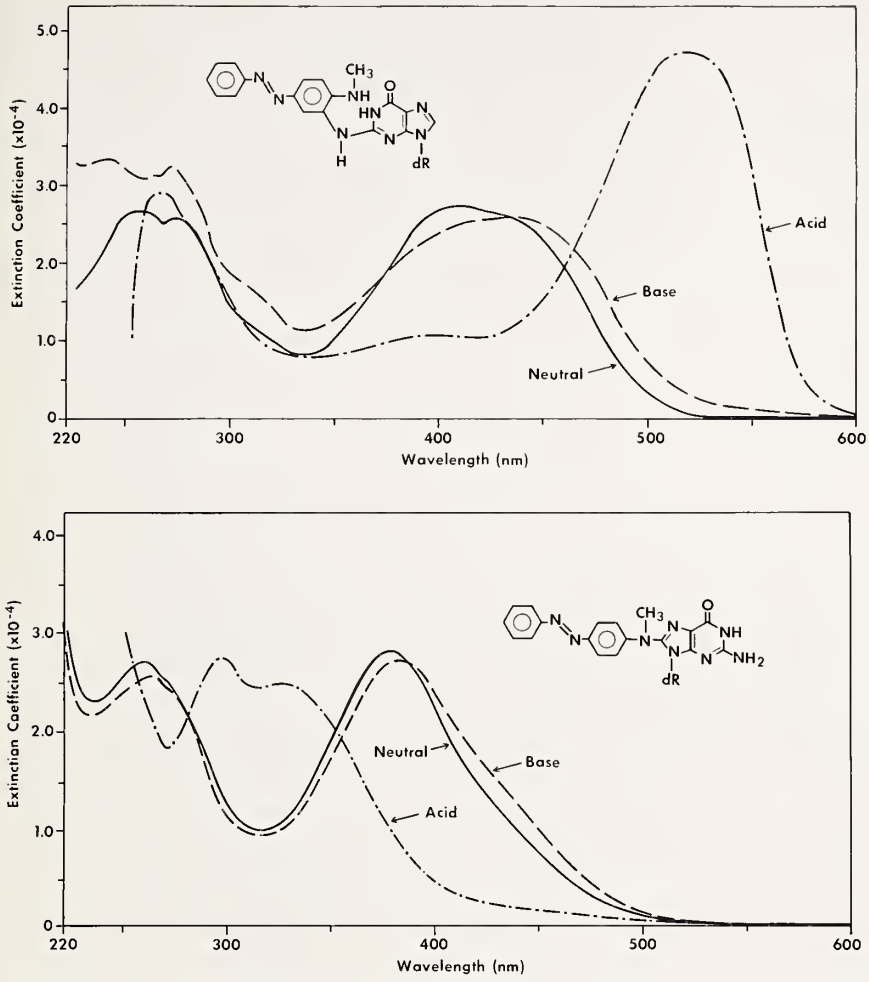
The UV and visible spectra on each of the products supported these assignments. Adduct II had a neutral UV spectrum consistent with a deoxyguanosinyl product with absorbances at 255 and 275 nm, but in contrast to adduct IV, this compound had an intense acid dye absorbance at 520 nm (text-fig. 4, upper portion). This absorbance intensity indicated that in adduct IV, the covalent linkage to dG was not through the amine nitrogen of MAB. Adduct IV in neutral solution had absorbances of 260 and 275 nm compatible with a dG adduct (text-fig. 4, lower portion). Moreover, this compound failed to absorb at 520 nm in acidic solution, which implied that the covalent bond must be through the amine nitrogen of MAB.

We obtained the pK_a of each of the adducts to provide additional information on the location of substitution of the dG. Both products had an acidic and a basic pK_a (text-fig. 5). Thus substitution through the deoxyguanosinyl N^1 or O^6 position was impossible because in such a situation a basic pK_a would not have been present.

We used ^{1}H-NMR spectroscopy to provide final structural proof on the adducts. The salient features of the spectrum for adduct IV were as follows (text-fig. 6A): Because

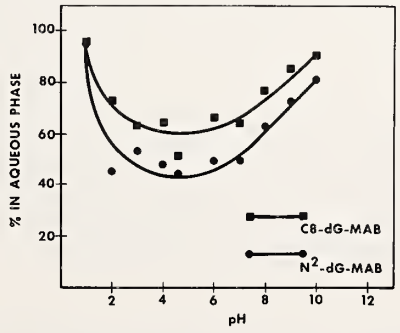


TEXT-FIGURE 3.—Electron-impact mass spectra of MAB-DNA adducts II and IV after silylation with pyridine-BSTFA (1:4) at 60° C for 1 hr. *Upper:* adduct II; *lower:* adduct IV. A mixture resulted from the silylation procedure; *m/z* 764 and 692 represent species that have acquired 4 and 3 silyl groups, respectively. Some depurination also occurred during the derivatization as indicated by the presence of *m/z* 576 (guanine-TMS_3).



TEXT-FIGURE 4.—UV spectra of MAB-DNA adducts II (*upper frame*) and IV (*lower frame*). Spectra were recorded in the following solvents: neutral: 50 mM sodium citrate, pH 7, 35% methanol; acid: 48% formic acid, 35% methanol; base: 100 mM sodium hydroxide, 35% methanol.

each of the aromatic and dR protons could be accounted for, MAB substitution had to be through either the azo linkage or the amine nitrogen. However, inasmuch as the N-methyl moiety appeared as a singlet and the MAB amine proton was absent, substitution must be through the MAB amine nitrogen. The C-8 proton of dG was absent but both N^2 protons were present. These data and the other spectroscopic evidence dictated that adduct IV was *N*-(dG-8-yl)-MAB. As final proof, this spectrum was identical to that of a product obtained from reacting *N*-BzO-MAB with 2′-dG (text-fig. 6B). This latter compound has been unequivocally established by Lin et al. (*24*) to be *N*-(dG-8-yl)-MAB.

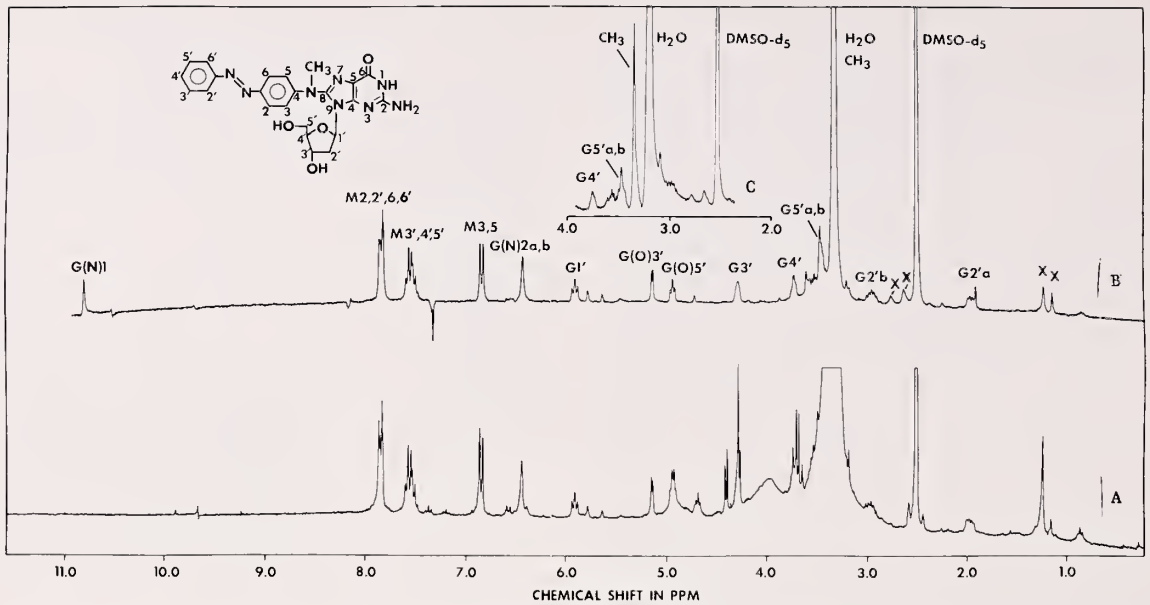


TEXT-FIGURE 5.—Partitioning of MAB-DNA adducts as a function of pH. Organic phase used with adduct II (dG-N^2-MAB) was 10% *n*-butanol in diethyl ether, whereas that used with adduct IV (dG-C8-MAB) was diethyl ether. The partitioning procedure of Moore and Koreeda (*21*) was followed.

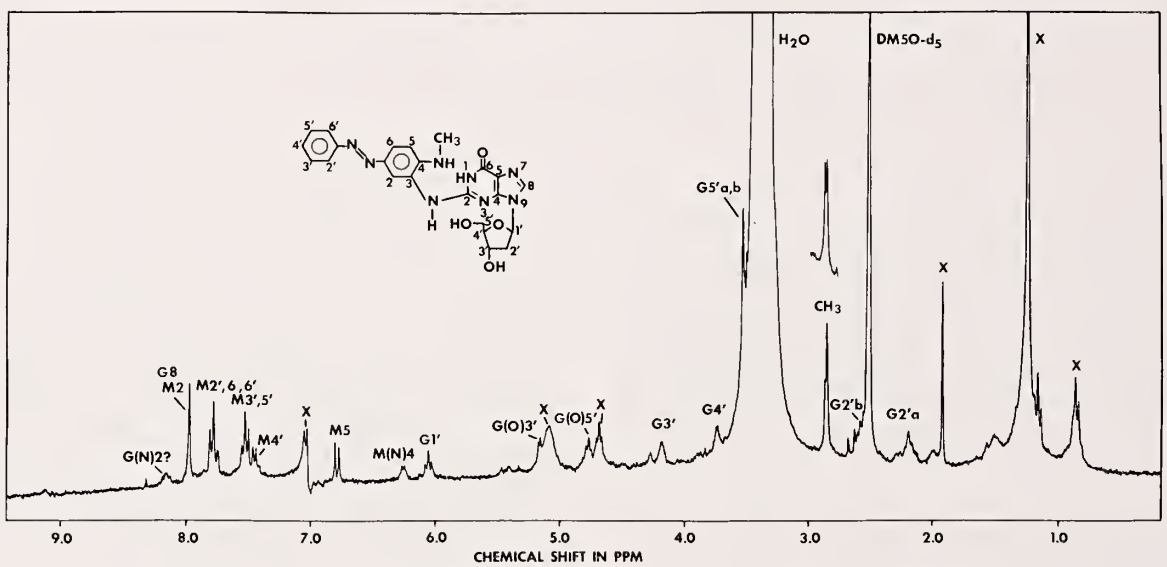
The NMR spectrum of adduct II contained a number of important features (text-figs. 7, 8). The fact that the amine methyl group appeared as a doublet and the amine proton as a quartet indicated that the dG was substituted through the aromatic rings of the MAB. To determine the exact site of substitution, we assigned the resonances of the parent compound MAB by performing experiments on homonuclear decoupling and nuclear Overhauser effect. When the spectra of MAB and adduct II were compared, we deduced that the proton at C-3 was absent; therefore, substitution must have occurred through this position. In addition, the NMR spectrum for adduct II contained resonances for all the dR protons and for the C-8 guanine proton. Absent, however, was a 2-proton resonance that could be assigned to the N^2 protons of dG (anticipated position, 6.5 ppm). The only structure compatible with this data was 3-(dg-N^2-yl)-MAB.

In Vivo Binding of *N*-Methyl-4-aminoazobenzene to Rat Liver DNA

To investigate the extent and nature of MAB-DNA binding in vivo, we treated male Sprague-Dawley rats



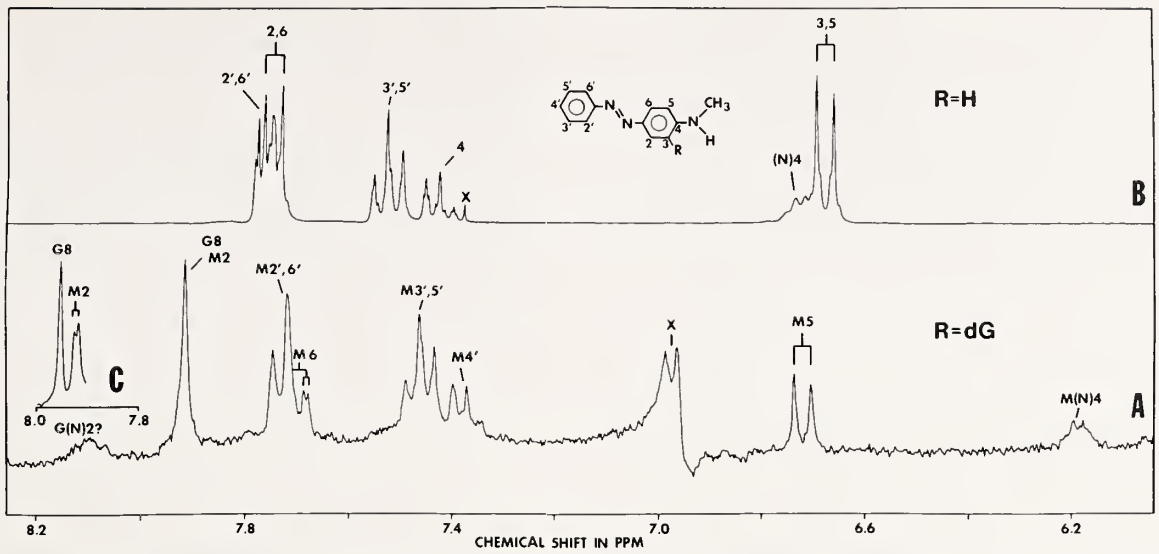
TEXT-FIGURE 6.—Assignments of *N*-(dG-8-yl)-MAB in DMSO-d_6 and 270-MHz proton NMR spectra. The M refers to the protons on the MAB moiety, G to those associated with dG. A) MAB-DNA adduct IV obtained by reaction of *N*-BzO-MAB with calf thymus DNA. Conditions: 1,000 60°-pulses at 7.6-sec intervals, 22° C. B) Adduct obtained from reaction of *N*-BzO-MAB with 2'-dG. Conditions: 20,000 80°-pulses at 8.7-sec intervals, 22° C. C) Indentical to B, except sample temperature was raised to 40° C; this caused water resonance to move upfield, which revealed the N-methyl peak.



TEXT-FIGURE 7.—Assignments of 37 μg MAB-DNA adduct II, 3-(dG-*N*²-yl)-MAB, in 100 μl DMSO-d_6 and 270-MHz proton NMR spectra. The M refers to protons on the MAB moiety, G to those on the dG. That the G(N)1 proton (anticipated position 10.5 ppm) was not detected may be due to the presence of salt in the sample. Resonance at 7.0 ppm is an artifact associated with the quadrature phase detection system. The insert of the N-methyl doublet was obtained from another preparation of this adduct. Conditions: 26,000 80°-pulses at 1.6-sec intervals, 22°C.

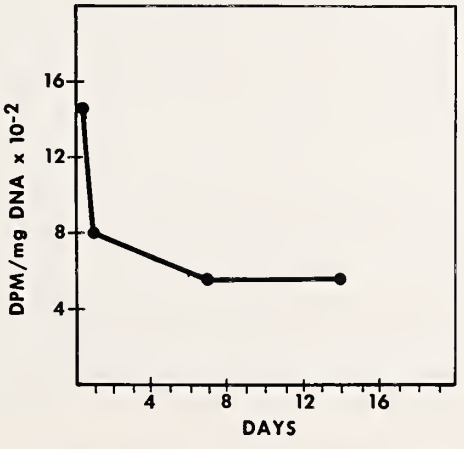
orally with (prime ring-^{3}H)-MAB. Then 8 hours, or 1, 7, or 14 days afterward, the animals were killed, their livers excised, and the carcinogen-bound DNA was isolated. Analysis of the DNA revealed maximum binding at the initial time point followed by a precipitous loss in radioactivity during the first 24 hours (text-fig. 9). Thereafter, the loss was less severe and, by 7 days, a constant binding level was reached with 25% of the radioactivity remaining.

The loss of radioactivity could represent one of two phenomena: either a general removal of all adducts or a

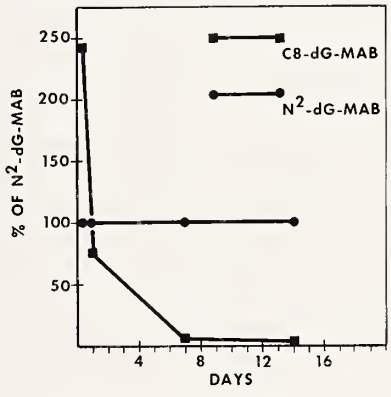


TEXT-FIGURE 8.—Assignments of 3-(dG-N^2-yl)-MAB(A) and MAB(B) in DMSO-d_6 270-HMz proton NMR spectra. A) Expansion of aromatic region of text-figure 7. B) Aromatic region of MAB. Conditions: 24 60°-pulses at 4.7-sec intervals, 22° C, 10 mg in 500 μl. C) Same as B except that D_2O had been added to sample. This caused G8 and M2 to diverge slightly and clearly demonstrated meta coupling in M2.

preferential loss of particular adducts. To resolve this question, we enzymatically hydrolyzed the DNA and separated the MAB adducts by HPLC. In the 8-hour sample, two adducts were detected. These cochromatographed with *N*-(dG-8-yl)- and 3-(dG-N^2-yl)-MAB. Initially, the C-8-substituted adduct was the major product and accounted for about 70% of the bound material (text-fig. 10). However, by 24 hours its concentration was nearly identical to the N^2-substituted product, and by 7 days it could no longer be detected. The N^2-substituted adduct, meanwhile, appeared to remain at a constant level throughout the entire sampling period.



TEXT-FIGURE 9.—Binding of MAB to rat liver DNA in vivo. Following treatment, animals were killed at either 8 hr, 1, 7, or 14 days. DNA was isolated and the total binding level was established. If no tritium was lost during metabolic activation or binding, 1,000 dpm represent a binding level of 1 adduct per 5.3×10^5 nucleotides.



TEXT-FIGURE 10.—Formation and persistence of individual MAB-DNA adducts in rat liver in vivo. ● = an adduct which cochromatographed with 3-(dG-N^2-yl)-MAB and remained at a constant level through 2 wk. ■ = an adduct which cochromatographed with *N*-(dG-8-yl)-MAB and was initially the major product. However, it could not be detected 7 days following treatment.

DISCUSSION

In a 1967 report on the persistent binding of DAB to rat liver DNA, Warwick and Roberts (*25*) found that, although 60% of the radioactivity was lost during the first week after treatment, the residual 40% remained permanently bound. Similar observations have also been reported by Chauveau et al. (*26*). In the ensuing years, the persistent binding of the carcinogenic aromatic amides, 2-fluorenylacetamide, and 4-acetylamino-4′-fluorobiphenyl to DNA has been established (*27–30*). For each of these carcinogens, two analogous arylamidated dG adducts in

DNA have been characterized. One is arylamide-N-substituted through the C-8 position of dG, whereas the other is arylamide ortho-substituted through the dG N^2 atom (*30, 31*). The C-8 lesions have been demonstrated to cause significant perturbation of the DNA helix (*32-41*), and this may account for their rapid removal from rat liver DNA in vivo (*29, 30, 42*). However, the N^2 adducts are stable persistent lesions (*29, 30, 42*), probably due to their location within the minor groove of the DNA helix which eliminates any significant steric distortions (*43*).

Lin et al. (*17*) established that a C-8 adduct, i.e., *N*-(dG-8-yl)-MAB was formed in rat liver DNA in vivo after MAB was administered. They found that this adduct represented approximately 40% of the bound radioactivity and remained constant from 15 through 46 hours (*17*); our results are compatible with theirs. Although we found the C-8 product to be the dominant adduct at 8 hours, it represented only 50% of the radioactivity by 24 hours. The adduct, however, continued to be lost, and by 7 days it could not be detected. This loss in *N*-(dG-8-yl)-MAB parallels the loss in total radioactivity originally observed by Warwick and Roberts (*25*). Cory-Pauling-Koltum space-filling models obtained from Ealing Corp. (South Natick, Mass.) of *N*-(dG-8-yl)-MAB indicate that it should have considerable steric interactions with the double helix. Based on the above reasoning, this lesion should be readily removed from DNA in vivo.

We also detected a second MAB-DNA adduct in rat liver in vivo; in contrast to *N*-(dG-8-yl)-MAB, this product appeared to be persistent. This adduct, which cochromatographed with one of the products obtained from reacting *N*-BzO-MAB with DNA in vitro, was characterized by a number of spectroscopic techniques and established to be 3-(dG-N^2-yl)-MAB. The space-filling models suggest that this adduct need not perturb the double helix (fig. 1) but rather that it may reside within the minor groove of the DNA helix. Similar stereochemistry has been proposed for other N^2 carcinogen adducts (*43*), although this is by no means the only possible structure (*44, 45*). Nevertheless, this lack of steric interaction may explain why it is not removed from rat liver in vivo.

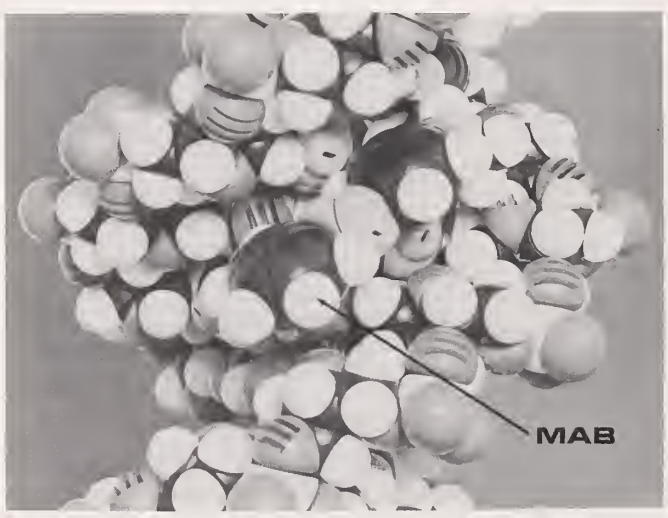


FIGURE 1.—CPK space filling model of 3-(dG-N^2-yl)-MAB situated in the minor groove of the DNA helix.

ADDENDUM:

Our more recent studies indicated the formation of an additional MAB-DNA adduct after repeated administration of MAB. This adduct cochromatographs with peak 5 (text-fig. 1) and has been identified through chemical and spectroscopic studies as 3-(deoxyadenosine-N^6-yl)-MAB (*46*).

REFERENCES

(*1*) KINOSITA R: Studies on the cancerogenic substances. Jpn Pathol Soc Trans 27:665-725, 1937

(*2*) MILLER JA, BAUMANN CA: The carcinogenicity of certain azo dyes related to *p*-dimethylaminoazobenzene. Cancer Res 5:227-234, 1945

(*3*) KINOSITA R: Studies on the cancerogenic azo and related compounds. Yale J Biol Med 12:287-300, 1940

(*4*) SUGIURA K, HALTER CR, KENSLER CJ, et al: Observations on rats fed with compounds related to dimethylaminoazobenzene. Cancer Res 5:235-238, 1945

(*5*) MUELLER GC, MILLER JA: The reductive cleavage of 4-dimethylaminoazobenzene by rat liver: Reactivation of carbon dioxide treated homogenates by riboflavin-adenine dinucleotide. J Biol Chem 185:145-154, 1950

(*6*) LU AY, KUNTZMAN R, CONNEY AH: The liver microsomal hydroxylation enzyme system. *In* Frontiers of Gastrointestinal Research, vol 2 (van der Reis L, ed). Basel: S Karger, 1976, pp 1-31

(*7*) MILLER JA, MILLER EC: The carcinogenic aminoazo dyes. Adv Cancer Res 1:339-396, 1953

(*8*) MILLER EC, KADLUBAR FF, MILLER JA, et al: N-Hydroxy metabolites of *N*-methyl-4-aminoazobenzene and related dyes as proximate carcinogens in the mouse and rat. Cancer Res 39:3411-3418, 1979

(*9*) SATO K, POIRIER LA, MILLER JA, et al: Studies on the N-hydroxylation and carcinogenicity of 4-aminoazobenzene and related compounds. Cancer Res 26:1678-1687, 1966

(*10*) ARCOS JC, ARGUS MF: Chemical Induction of Cancer, vol IIB. New York: Academic Press, 1974, pp 140-216

(*11*) DALY JW, JERINA DM, WITKOP B: Arene oxides and the NIH shift: The metabolism, toxicity and carcinogenicity of aromatic compounds. Experientia 28:1129-1149, 1972

(*12*) YANG SK, CHOU MW, WEEMS HB, et al: Enzymatic formation of an 8,9-diol from 8-methylbenz[*a*]anthracene. Biochem Biophys Res Commun 90:1136-1141, 1979

(*13*) KADLUBAR FF, MILLER JA, MILLER EC: Microsomal N-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and reactivity of *N*-hydroxy-*N*-methyl-4-aminozobenzene. Cancer Res 36:1196-1206, 1976

(14) _____: Hepatic metabolism of *N*-hydroxy-*N*-methyl-4-aminoazobenzene and other N-hydroxy arylamines to reactive sulfuric acid esters. Cancer Res 36:2350-2359, 1976

(*15*) LABUC GE, BLUNCK JM: Metabolic activation of the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene by a rat liver cell-free system. Biochem Pharmacol 28: 2367-2373, 1979

(*16*) BLUNCK JM, CROWTHER CE: Enhancement of azo dye carcinogenesis by dietary sodium sulfate. Eur J Cancer 11: 23-31, 1975

(*17*) LIN JK, MILLER JA, MILLER EC: Structures of hepatic nucleic acid-bound dyes in rats given the carcinogen *N*-methyl-4-aminoazobenzene. Cancer Res 35:844–850, 1975

(*18*) LIN JK, MILLER JA: Synthesis of the hepatocarcinogen *N*-methyl-4-aminoazobenzene with tritium in the prime ring. J Labeled Compds 5:257–260, 1969

(*19*) BELAND FA, DOOLEY KL, CASCIANO DA: Rapid isolation of carcinogen-bound DNA and RNA by hydroxyapatite chromatography. J Chromatogr 174:177–186, 1979

(*20*) KADLUBAR FF, MILLER JA, MILLER EC: Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res 37:805–814, 1977

(*21*) MOORE PD, KOREEDA M: Application of the change in partition coefficient with pH to the structure determination of alkyl substituted guanosines. Biochem Biophys Res Commun 73:459–464, 1976

(*22*) POIRIER LA, MILLER JA, MILLER EC, et al: *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene: Its carcinogenic activity in the rat and its reactions with proteins and nucleic acids and their constituents in vitro. Cancer Res 27: 1600–1613, 1967

(*23*) WISLOCKI PG, MILLER JA, MILLER EC: The carcinogenic and electrophilic activities of N-benzoyloxy derivatives of *N*-methyl-4-aminoazobenzene and related dyes. Cancer Res 35:880–885, 1975

(24) LIN J-K, SCHMALL B, SHARPE ID, et al: N-Substitution of carbon 8 in guanosine and deoxyguanosine by the carcinogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene in vitro. Cancer Res 35:832–843, 1975

(*25*) WARWICK GP, ROBERTS JJ: Persistent binding of butter yellow metabolites to rat liver DNA. Nature 213:1206–1207, 1967

(*26*) CHAUVEAU J, DECLOITRE F, BENOIT A, et al: Relationship between azodye carcinogenicity and induction of repairable damage of DNA in rat liver. Colloques Int (CNRS) 256:417–430, 1977

(*27*) EPSTEIN SM, MCNARY J, BARTUS B, et al: Chemical carcinogenesis: Persistence of bound forms of 2-fluorenylacetamide. Science 162:907–908, 1968

(*28*) IRVING CC, VEAZEY RA: Persistent binding of 2-acetylaminofluorene to rat liver DNA in vivo and consideration of the mechanisms of binding of *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids. Cancer Res 29:1799–1804, 1969

(*29*) KRIEK E: Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA in vivo. Cancer Res 32: 2042–2048, 1972

(*30*) KRIEK E, HENGEVELD GM: Reaction products of the carcinogen *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl with DNA in liver and kidney of the rat. Chem Biol Interact 21:179–201, 1978

(*31*) WESTRA JG, KRIEK E, HITTENHAUSEN H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149–164, 1976

(*32*) KAPULER AM, MICHELSON AM: The reaction of the carcinogen *N*-acetoxy-2-acetylaminofluorene with DNA and other polynucleotides and its stereochemical implications. Biochim Biophys Acta 232:436–450, 1971

(*33*) FUCHS R, DAUNE M: Changes of stability and conformation of DNA following the covalent binding of a carcinogen. FEBS Lett 14:206–208, 1971

(*34*) _____: Physical studies of deoxyribonucleic acid after covalent binding of a carcinogen. Biochemistry 11:2659–2666, 1972

(*35*) _____: Physical basis of chemical carcinogenesis by *N*-2-fluorenylacetamide derivatives and analogs. FEBS Lett 34:295–298, 1973

(*36*) LAVINE AF, FINK LM, WEINSTEIN IB, et al: Effects of *N*-2-acetylaminofluorene modification on the conformation of nucleic acids. Cancer Res 34:319–327, 1974

(*37*) FUCHS RP, DAUNE MP: Dynamic structure of DNA modified with the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. Biochemistry 13:4435–4440, 1974

(*38*) METZGER G, DAUNE MP: In vitro binding of *N*-acetoxy-*N*-2-acetylaminofluorene to DNA in chromatin. Cancer Res 35:2738–2742, 1975

(*39*) FUCHS RP, LEFEVRE JF, POUYET J, et al: Comparative orientation of the fluorene residue in native DNA modified by *N*-acetoxy-*N*-2-acetylaminofluorene and two 7-halogeno derivates. Biochemistry 15:3347–3351, 1976

(*40*) WEINSTEIN IB, GRUNBERGER D: Structural and functional changes in nucleic acids modified by chemical carcinogens. *In* Chemical Carcinogenesis, Part A (Ts'o P, DiPaolo J, eds.) New York: Marcel Dekker, 1974, pp 217–235

(*41*) GRUNBERGER D, WEINSTEIN IB: The base displacement model: An explanation for the conformational and functional changes in nucleic acids modified by chemical carcinogens. *In* Biology of Radiation Carcinogenesis (Yuhas JM, Tennant RW, Regan JD, eds). New York: Raven Press, 1976, pp 175–187

(*42*) BELAND FA, DOOLEY KL, EVANS FE, et al: Role of persistent DNA-bound residues of *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) in tumor induction. Proc Am Assoc Cancer Res 20:128, 1979

(*43*) BELAND FA: Computer-generated graphic models of the N^2-substituted deoxyguanosine adducts of 2-acetylaminofluorene and benzo[*a*]pyrene and the O^6-substituted deoxyguanosine adduct of 1-naphthylamine in the DNA double helix. Chem Biol Interact 22:329–339, 1978

(*44*) SWENSON DH, KADLUBAR FF: Properties of chemical mutagens and chemical carcinogens in relation to their mechanisms of action. *In* Microbial Testers Probing Carcinogenesis (Felkner IC, ed). New York: Marcel Dekker, 1981, pp 1–33

(*45*) KADLUBAR FF: A mutation hypothesis for N^2-substitution of guanine in DNA by chemical carcinogens. Chem Biol Interact 31:255–263, 1980

(46) TULLIS DL, KADLUBAR FF, STRAUB KM: Hepatic DNA adducts in the rat after multiple doses of the carcinogen, *N*-methyl-4-aminobenzene. Chem Biol Interact. In press

DNA Adducts Formed From *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene In Vitro and From *N,N*-Dimethyl-4-aminoazobenzene in Mouse Liver[1]

W. Gary Tarpley, James A. Miller, *and* Elizabeth C. Miller[2]

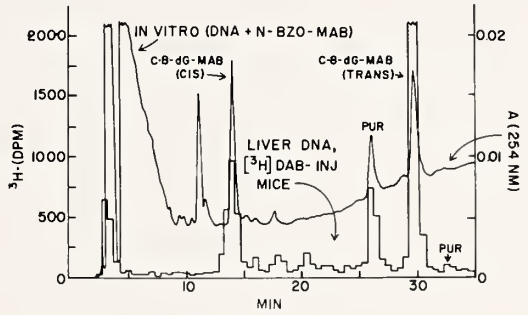
ABSTRACT—Reaction of *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene with [^{14}C]deoxyguanosine yielded a series of at least 10 dye derivatives separable by high-performance liquid chromatography. The major adduct, *N*-[deoxyguanosin-8-yl]-*N*-methyl-4-aminoazobenzene, was present as both *cis* and *trans* isomers. Similar series of adducts were obtained from enzymatic digests of DNA reacted in vitro with *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene or from hepatic DNA of (C57BL/6 × C3H/He)F_1 males given injections at 12 days of age of [prime ring-^{3}H]*N,N*-dimethyl-4-aminoazobenzene. The concentrations of the major adduct and of the second most prominent adduct in the hepatic DNA, after correction for liver growth, were approximately 30 and 70% of the initial values after 10 days; thus the second adduct was poorly removed in comparison to the major adduct.—Natl Cancer Inst Monogr 58: 163-164, 1981.

We investigated the formation of DNA adducts in vitro after reaction of the model ultimate carcinogen *N*-BzO-MAB and in vivo in mouse liver after administration of [prime ring-^{3}H]DAB or [prime ring-^{3}H]MAB. In earlier studies from our laboratory by Lin et al. (*1, 2*), the major adduct in vitro and in vivo was characterized as *N*-(deoxyguanosin-8-yl)-*N*-methyl-4-aminoazobenzene. Analysis by HPLC of the reaction products permitted much greater resolution than was possible with the thin-layer chromatography used earlier. Thus the reaction of *N*-BzO-MAB with dG yields 10 separable dye derivatives, of which 2 account for about 80% of the products.

Similarly, as shown in text-figure 1, the reaction of *N*-BzO-MAB and DNA with subsequent enzymatic digestion (*Neurospora crassa* endonuclease, DNase 1, alkaline phosphatase, venom, and spleen phosphodiesterase) yields a number of adducts. Most of these adducts have retention times similar to the products obtained on reaction with dG. Two of the products obtained by HPLC from either the dG reaction mixture or the DNA digest are the *N*-(dG-8-yl)-MAB adducts. The two products are interconvertible, especially in the presence of light, and are apparently the *cis* and *trans* isomers. Other adducts on these chromatograms probably also exist as *cis-trans* pairs. The adducts in this text-figure all appear to be purine adducts on the basis of the reaction of *N*-BzO-MAB with *Escherichia coli* DNA in which the purines are labeled with ^{14}C.

This text-figure also shows the distribution of ^{3}H in the nucleoside adducts obtained after digestion of the hepatic DNA from 12-day-old male (C57BL/6 × C3H/He)F_1 mice killed 24 hours after a single injection of 120 mg/kg of [^{3}H]-DAB. The ^{3}H in these adducts generally co-chromatographs with the 254-nm absorbance of the products obtained from the in vitro reaction with DNA. These hybrid mice develop hepatomas in high incidence by 10 months, if a total of 1.5 mmol/kg body wt of MAB or DAB is administered in 4 doses prior to weaning.

The 2 isomers of *N*-(dG-8-yl)-MAB account for about 40% of the ^{3}H in the mouse liver DNA. A second nucleoside product, presumably a purine-containing adduct, has a retention time of 26 minutes and accounts for about 10% of the dye adducts in the DNA of the mouse livers (text-fig. 2). The remaining adducts individually account for 1-5% of the ^{3}H in the DNA. In a preliminary

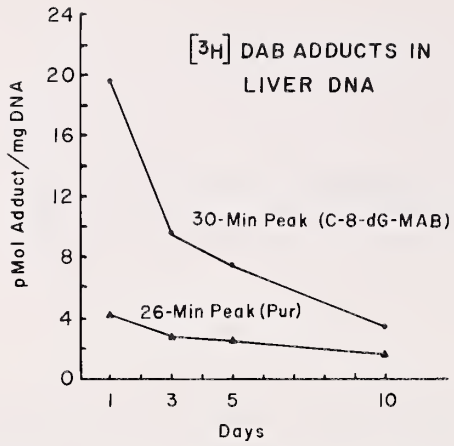


TEXT-FIGURE 1.—Cochromatography of the DNA-dye adducts formed by reaction of *N*-BzO-MAB in vitro and from DAB in vivo. Enzymatic digests (to the nucleoside level) of 0.05 mg *N*-BzO-MAB-reacted calf thymus DNA and 0.075 mg hepatic DNA isolated 24 hr after a single ip injection of [prime ring-^{3}H]DAB into each 12-day-old (C57BL/6 × C3H/He)F_1 male were extracted three times with 2 vol of water-saturated 1-butanol. The butanol was removed at 40° C under reduced pressure, and the residues were dissolved together in 25% acetonitrile in water for HPLC on a μBondapak C_{18} reverse-phase column. The column was developed with a linear gradient of 25-40% acetonitrile-water for 35 min at the rate of 1 ml/min. N-BZO-MAB = *N*-benzoyloxy-MAB; C-8-dG-MAB = *N*-(dG-8-yl)-MAB; DPM=disintegrations/min; INJ = injected.

Abbreviations: MAB = *N*-methyl-4-aminoazobenzene; N-BzO-MAB = *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene; DAB = *N,N*-dimethyl-4-aminoazobenzene; dG = deoxyguanosin(e); HPLC = high-performance liquid chromatography.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7-9, 1979.

[2] McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706. *Address reprint requests to* Dr. Elizabeth C. Miller.



TEXT-FIGURE 2.—Loss of 2 major dye adducts from hepatic DNA as a function of time. Each (C57BL/6 × C3H/He)F_1 male mouse received a single ip injection of [prime ring-^{3}H]DAB (120 mg/kg body wt; 2 Ci/mmol). One, 3, 5, or 10 days later the hepatic nucleic acids were isolated, enzymatically digested to nucleosides, and extracted three times with 2 vol of water-saturated 1-butanol. The butanol extracts were evaporated to dryness at 40° C under reduced pressure, and the residues were dissolved in 20% acetonitrile in water for analysis by HPLC on a μBondapak C_{18} reverse-phase column with step gradients of 20%, 25%, 30%, and 35% acetonitrile–water for 15, 10, 10, and 15 min, respectively. The flow-rate was 1 ml/min. *Note* concentrations of the 2 major dye adducts in the DNA.

study, we also found a similar adduct profile for the liver DNA of rats administered single doses of 120 mg [^{3}H]-MAB/kg.

The *N*-(dG-8-yl)-MAB disappeared at a faster rate from the mouse liver DNA than did the 26-minute product. Ten days after the administration of dye, the concentrations (picamoles/milligram DNA) of the C-8 adduct and the 26-minute product were about 17 and 40%, respectively, of their concentrations at 24 hours. The real decreases in concentration of both adducts after 10 days are approximately 60% less, due to the 1.6-fold increase in liver size during this period. By analogy to the DNA adducts formed from 2-fluorenylacetamide (*3*) and consistent with Dr. Beland's data (*4*), the product at 26 minutes is probably 3-(dG-N^2-yl)-MAB. These adducts are being investigated further in our laboratory.

REFERENCES

(*1*) LIN J-K, SCHMALL B, SHARPE ID, et al: N-Substitution of carbon 8 in guanosine and deoxyguanosine by the carcinogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene in vitro. Cancer Res 35:832–843, 1975

(*2*) LIN J-K, MILLER JA, and MILLER EC: Structures of hepatic nucleic acid-bound dye in rats given the carcinogen *N*-methyl-4-aminoazobenzene. Cancer Res 35:844–850, 1975

(*3*) WESTRA JG, KRIEK E, HITTENHAUSEN H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149–164, 1976

(*4*) BELAND FA, TULLIS DL, KADLUBAR FF, et al: Identification of the DNA adducts formed in vitro from *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene and in rat liver in vivo after administration of *N*-methyl-4-aminoazobenzene. Natl Cancer Inst Monogr 58:153–161, 1981.

Significance of Metabolic Activation and Binding to Nucleic Acids of Aminostilbene Derivatives In Vivo[1,2,3]

H.-G. Neumann[4,5]

ABSTRACT—Comparison of metabolite binding of several aminostilbene-related compounds to rat liver macromolecules in vivo supported the concept that metabolic activation is a prerequisite for biologic activity. Carcinogenic *trans*-4-dimethylaminostilbene and *trans*-4-acetylaminostilbene bound more strongly to DNA than the biologically less active *cis*-4-acetylaminostilbene and 4-dimethylaminobibenzyl by more than ten times. Hydroxamic acid esters did not appear to be the major metabolites which ultimately reacted with nucleic acids. The primary biochemical lesions are not correlated with tissue susceptibility. Total binding of *trans*-4-dimethylaminostilbene metabolites to nucleic acids was highest in the liver, about one-fifth of that total amount in the kidney, less than one-fifth in the lung and glandular stomach (which is the target tissue for acute toxicity), and still less in the forestomach and Zymbal's gland, the tissue in which tumors arise after repeated administration of test compounds to female Wistar rats. In the nontarget tissues, i.e., liver and kidney, nucleic acid binding was not only initially high but also persistent. Therefore, the exposure-related, primary biochemical lesions could not be linked to the biologic lesion. Tissue-specific parameters, other than those related to metabolic activation, are proposed to determine the biologic effect.—Natl Cancer Inst Monogr 58: 165–171, 1981.

The carcinogenic aromatic amine *trans*-DAS attracted our attention as a suitable model compound for several reasons: It is acutely toxic and strongly carcinogenic, and it produces tumors with high tissue specificity (*1–5*). Moreover, it can be highly and specifically labeled with tritium, and the labeling also makes the structurally closely related, but biologically less active, compounds *cis*-DAS and DABB accessible (*6, 7*). Results obtained recently in an analysis of the fate of these compounds in female Wistar rats will be discussed. Major emphasis is placed on consideration of the role of nucleic acid-bound metabolites in the carcinogenic activity of *trans*-DAS.

Abbreviations: *trans*(*cis*)-DAS = *trans*(*cis*)-4-dimethylaminostilbene; DABB = 4-dimethylaminobibenzyl; *trans*(*cis*)AAS = *trans* (*cis*)-4-acetylaminostilbene; fmol = femtomole(s).

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported by the Deutsche Forschungsgemeinschaft.

[3] This paper is dedicated to Professor Elwood V. Jensen.

[4] Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Strasse 9, 8700 Würzburg, Federal Republic of Germany.

[5] The data presented here were supplied by H. Baur, B. J. M. Gaugler, P. Marquardt, and R. Wirsing of the Institut für Pharmakologie und Toxikologie.

METABOLIC ACTIVATION

The overall metabolism of the three amines appears similar and involves oxidation to phenols and hydroxamic acids, which are excreted predominantly as sulfates and glucuronic acid conjugates (*8*). In addition, diols are formed from the stilbene double bond, which we interpret as an indication that a stilbene epoxide is an intermediate (*9*).

To support the present belief that covalent binding of reactive metabolites to DNA is causally related to cell transformation, one should correlate the extent of DNA binding with biologic activity. This correlation is seen if the 3 structurally related compounds are compared. After oral administration of the [^{3}H]-labeled test compounds, we (*10*) found that binding to proteins as well as to rRNA and DNA is considerably higher with carcinogenic *trans*-DAS or -AAS than with *cis*-AAS or DABB (table 1). Enzymatic hydrolysis of the nucleic acids and fractionation of the resulting nucleosides show that, in contrast to many other aromatic amines, a multitude of nucleoside adducts is formed with *trans*-DAS metabolites (*10*). Some of these adducts have been identified by comparison with synthetic adducts, which were obtained by the reaction of *trans*-*N*-acetoxy-*N*-AAS with nucleosides, homopolynucleotides, or nucleic acids as shown in text-figure 1 (*11, 12*). However, we (*13*) also determined that only some of the major in vitro adducts are formed in vivo in minute amounts (text-fig. 2), which indicated to us that hydroxamic acid esters contribute only little to the ultimate reactive *trans*-DAS metabolites in rat liver.

A similar conclusion may be drawn from mutagenicity testing of *trans*-aminostilbene derivatives in different strains of *Salmonella typhimurium*. As previously observed (*14*), *trans*-4-nitrosostilbene is the most mutagenic derivative; however, it still does not appear to be the ultimate one, nor does *N*-acetoxy-*N*-AAS. The results could best be interpreted if *N*-hydroxy-aminostilbene were the common intermediate between the compounds tested and the ultimate reactive form (*15*).

Inasmuch as metabolites of *trans*-DAS are more mutagenic and bind more strongly to nucleic acids than the parent compound, our findings support the general view that metabolic activation is a prerequisite for biologic activity. The differences between the highly active *trans*-DAS

TABLE 1.—*Binding (picomoles/milligram) of different aminostilbene derivatives to liver macromolecules*[a]

Macromolecule	Time after administration, hr	Compound administered			
		trans-DAS	*trans*-AAS	*cis*-AAS[b]	DABB
Proteins	5	42	79	20.5 (11.0)	11
	24	101	107	26.5 (13.7)	11
rRNA	5	3.5	11.7	1.5 (0.1)	0.2
	24	15.0	17.3	2.5 (0.4)	0.4
DNA	5	5.0		1.4	0.1
	24	15.4[c]	21.1	2.3 (0.0)	0.6

[a] ^{3}H-labeled compounds (25 μmol/kg) were administered orally to female Wistar rats. Material from 2 to 3 animals was pooled, and radioactivity was determined in duplicate (*10*).

[b] *cis*-AAS contained about 12% of the *trans*-isomer. *Values in parentheses* are corrected for this content.

[c] The dose in this experiment was 22 μmol/kg.

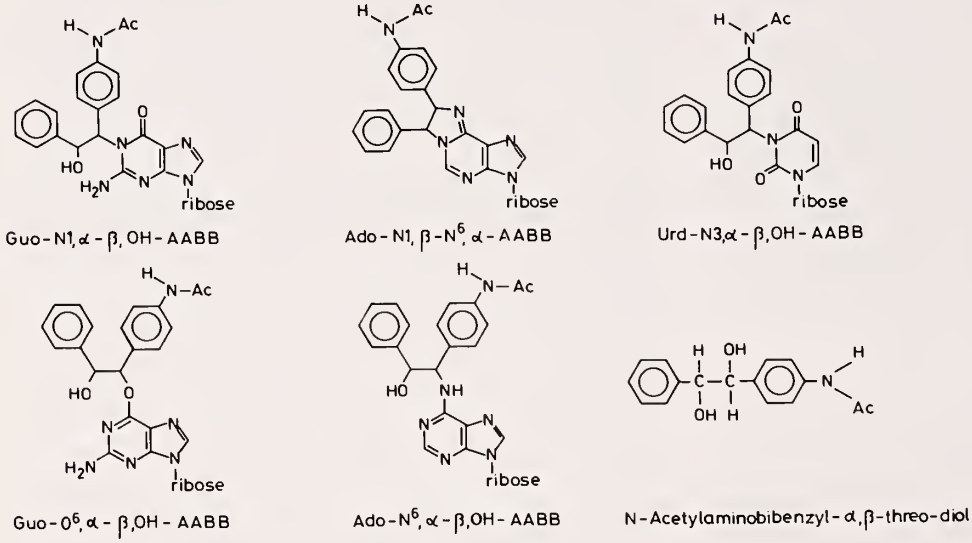
and the less active *cis*-DAS and DABB are likely to be explained by the higher chemical reactivity of the ultimate metabolites. In addition, higher local concentrations of certain metabolites, due to differential tissue affinities, contribute to this effect (*16*).

However, the concept of metabolic activation requires more than the formation of appropriately reactive metabolites. One would also expect that the extent of reaction correlates in some way with tissue susceptibility.

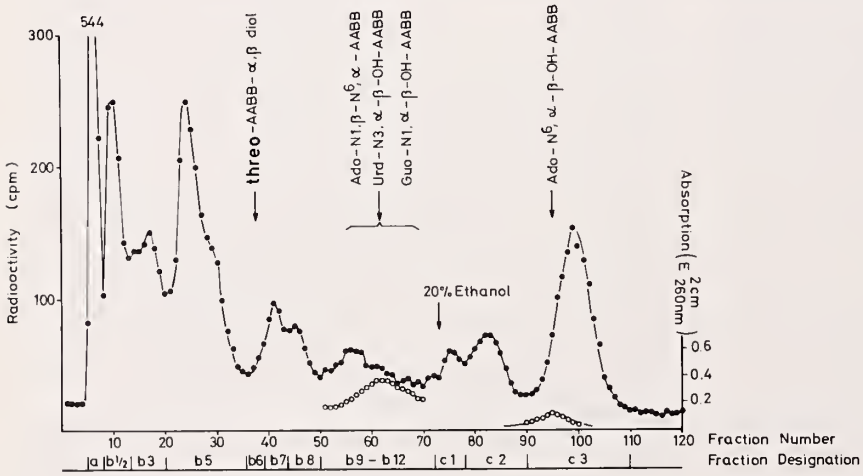
BINDING TO NUCLEIC ACIDS AND TISSUE SUSCEPTIBILITY

Trans-DAS selectively induces ear duct tumors in the rat after chronic feeding (*1, 2, 5,*); only occasionally are a few cholangiomas or some mammary tumors (with *trans*-AAS) observed (*17–19*). Thus the primary target tissue for chronic effects is the Zymbal's gland; at the most, liver could be considered a secondary target. Acute toxicity, which also requires metabolic activation, is manifested in the stomach and bone marrow. The liver is not affected even with lethal doses of *trans*-DAS, i.e., no substance-related morphologic alterations could be detected (*20*).

If reaction with cellular macromolecules is the prime prerequisite for acute and chronic effects, then tissue exposure to reactive metabolites, with resulting binding to proteins and nucleic acids, should reflect the tissue burden of biochemical lesions. With *trans*-DAS, target and non-



TEXT-FIGURE 1.—Structures of reference compounds (*11, 12*) available for comparison with hydrolysates from nucleic acids obtained from in vivo experiments. For further details, *see* (*10*).



TEXT-FIGURE 2.—Chromatography of RNA hydrolysates on Sephadex LH-20. Reference compounds indicated (*see* text-fig. 1) were added to an RNA hydrolysate obtained from rat liver rRNA after oral administration of [^{3}H]*trans*-DAS. Mixture was chromatographed on Sephadex LH-20 (*10*). Radioactivity eluting together with threo-AABB-α,β-diol; Ado-N1,β-N6,α-AABB; Urd-N3,α-β,OH-AABB; and Guo-N1,α-β,OH-AABB cochromatographed also on reruns on Sephadex LH-20 and on thin-layer chromatograms (*13*). In addition, fraction c1 cochromatographed with Guo-O6,α-β,OH-AABB.

target tissues cannot be differentiated in this respect. Twenty-four hours after administration of *trans*-DAS, binding to nucleic acids is highest in liver, roughly one-fifth of that in kidney, somewhat less in the lung and glandular stomach, and still less in the forestomach and Zymbal's gland. Binding to RNA and DNA is similar after 24 hours, whereas binding to proteins is consistently five to eight times higher than to nucleic acids.[4] Neither the glandular stomach nor Zymbal's gland appear notably exposed compared with definite nontarget tissues.

One possible explanation for the discrepancy between high initial binding and low tissue susceptibility is that primary lesions are rapidly repaired in nonsensitive tissues; this is not true for *trans*-DAS. The result of a repair experiment in which we analyzed total binding (proteins, rRNA, and DNA in liver and kidney at 2 and 4 wk after a single administration of *trans*-DAS) clearly showed that DNA-bound metabolites are slowly eliminated. The half-lives are 34 and 60 days in liver and kidney, respectively, as illustrated in text-figure 3.[4]

Another explanation could be obtained if predominant binding of either noncritical metabolites or binding to noncritical sites were measured. To evaluate this hypotheses, we are now conducting experiments in this laboratory to compare the pattern of nucleoside adducts from RNA and DNA in different tissues. However, it is difficult to imagine that the persistent binding of a bulky molecule, such as *trans*-aminostilbene, to several bases should not represent a biochemical lesion.

TISSUE-SPECIFIC METABOLIC ACTIVATION

Investigators have often claimed that the ultimate reactive metabolites are formed within the affected cell. Enzymes are thought to release critical intermediates close to the target molecules, and the susceptible tissue could be characterized by specific enzyme activities. We interpret our findings as indicating that the extent of nucleic acid binding of *trans*-DAS metabolites in extrahepatic tissues is not determined by local activation but primarily by enzymatic activation in the liver. This does not exclude the possibility that the final steps of the metabolic pathway occur within the cell but rather indicates that the extent of binding is unrelated to tissue-specific activation.

Support for this interpretation is derived from studies in which the dose dependence of metabolic activation is determined. Binding to macromolecules is correlated with the total concentration of metabolites only in liver tissue and not in the blood and stomach. In all three tissues, specific binding increases only threefold for a tenfold dose increase at 5 hours with the highest dose administered. Thus the ratio of liver:tissue binding remains constant, whereas the ratios of liver:total metabolites do not (*21*).

Another point of support is derived from studies in which binding to kidney macromolecules has been determined after different routes of administration (table 2). Total binding to DNA is similar after oral, ip, and iv administration of equal doses of *trans*-DAS.[4] Similarly, binding to plasma proteins and hemoglobin is independent of the route of administration (table 3), despite the fact that initial blood levels and, consequently, concentrations in extrahepatic tissues are significantly higher after iv injection than after oral or ip administration (Wirsing R, Neumann H-G: Unpublished results).

TABLE 2.—*Binding of metabolites in kidney 24 hr after administration of 250 nmol [^{3}H]*trans*-DAS/kg by different routes*[a]

Route of administration	Tissue concentration	Binding, fmol/mg			No. of determinations from No. of animals
		Protein	rRNA	DNA	
Oral	230	484	88	47	3/2
ip	209	410	59	35	2/3
iv	273	344	75	45	1/4

[a] Nucleic acids were isolated according to (*10*) or by a modified procedure with hydroxyapatite (Baur H: Unpublished results).

TABLE 3.—*Binding of metabolites to blood proteins 24 hr after administration of 250 nmol of [^{3}H]*trans*-DAS/kg by different routes*

Route of administration	Binding, fmol/mg[a]		No. of determinations
	Plasma proteins	Hemoglobin	
Oral	0.53 ± 0.13	0.99 ± 0.02	2
ip	0.63 ± 0.09	0.78 ± 0.33	4
iv	0.62 ± 0.06	0.77 ± 0.01	2

[a] Blood was processed according to (*22*).

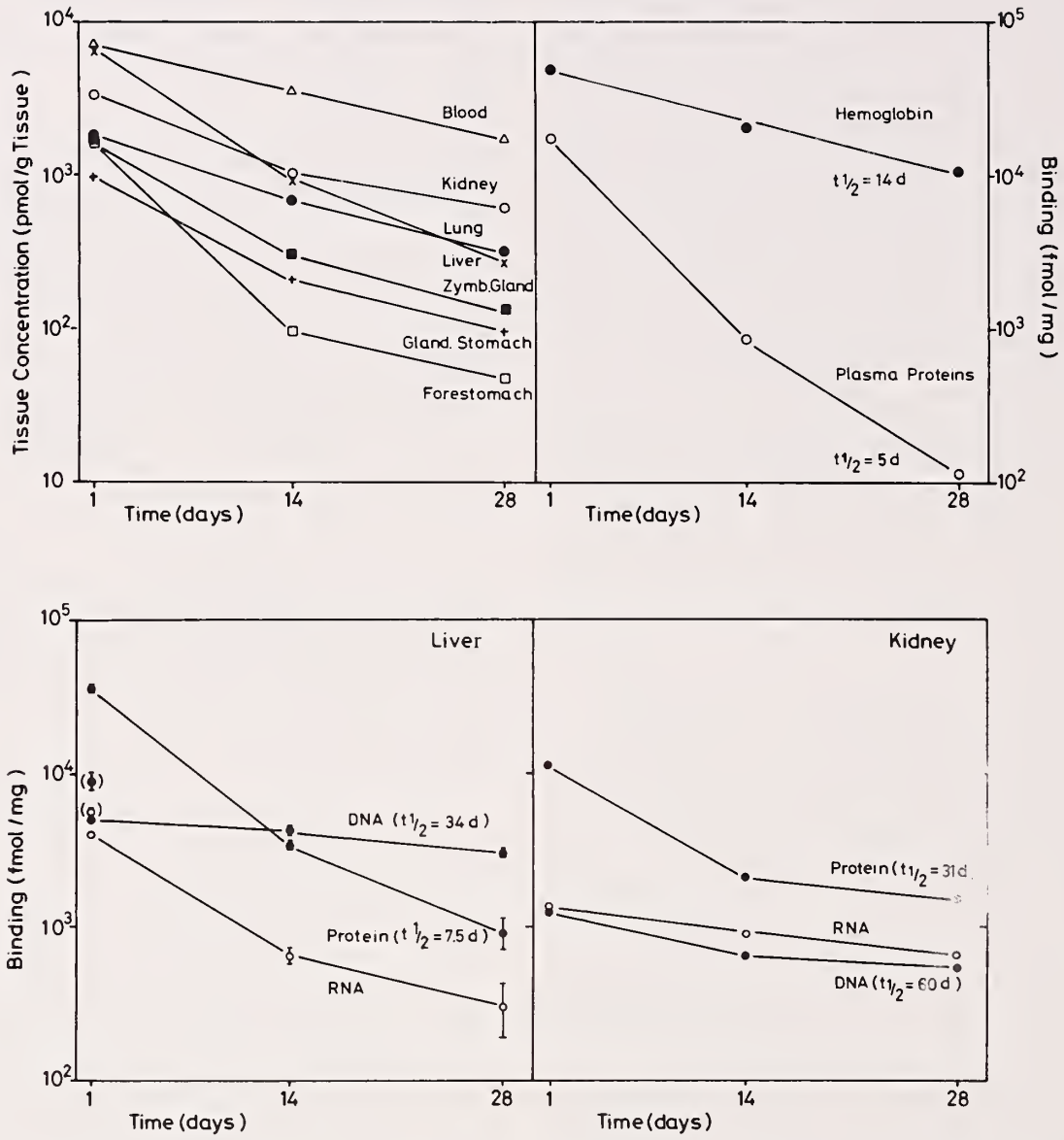
OTHER TISSUE-SPECIFIC PROPERTIES

Cells appear to be much more vulnerable in the stage of proliferation. With *trans*-DAS we find that the acutely toxic effects, as already mentioned, are seen in the glandular stomach and bone marrow. In the glandular stomach, it is the proliferating isthmus cells of the antrum which are primarily affected by toxic doses (*20*). These are the cells with the highest proliferative rate of the entire organ. The high proliferation rate of hematopoietic cells is well established, and Zymbal's gland, which is a holocrine gland, could also be expected to proliferate rapidly. On the other hand, acute lesions do not occur in the intestinal epithelium where proliferation is equally high. This finding indicates that susceptible cells proliferate but that not all rapidly proliferating cells are susceptible.

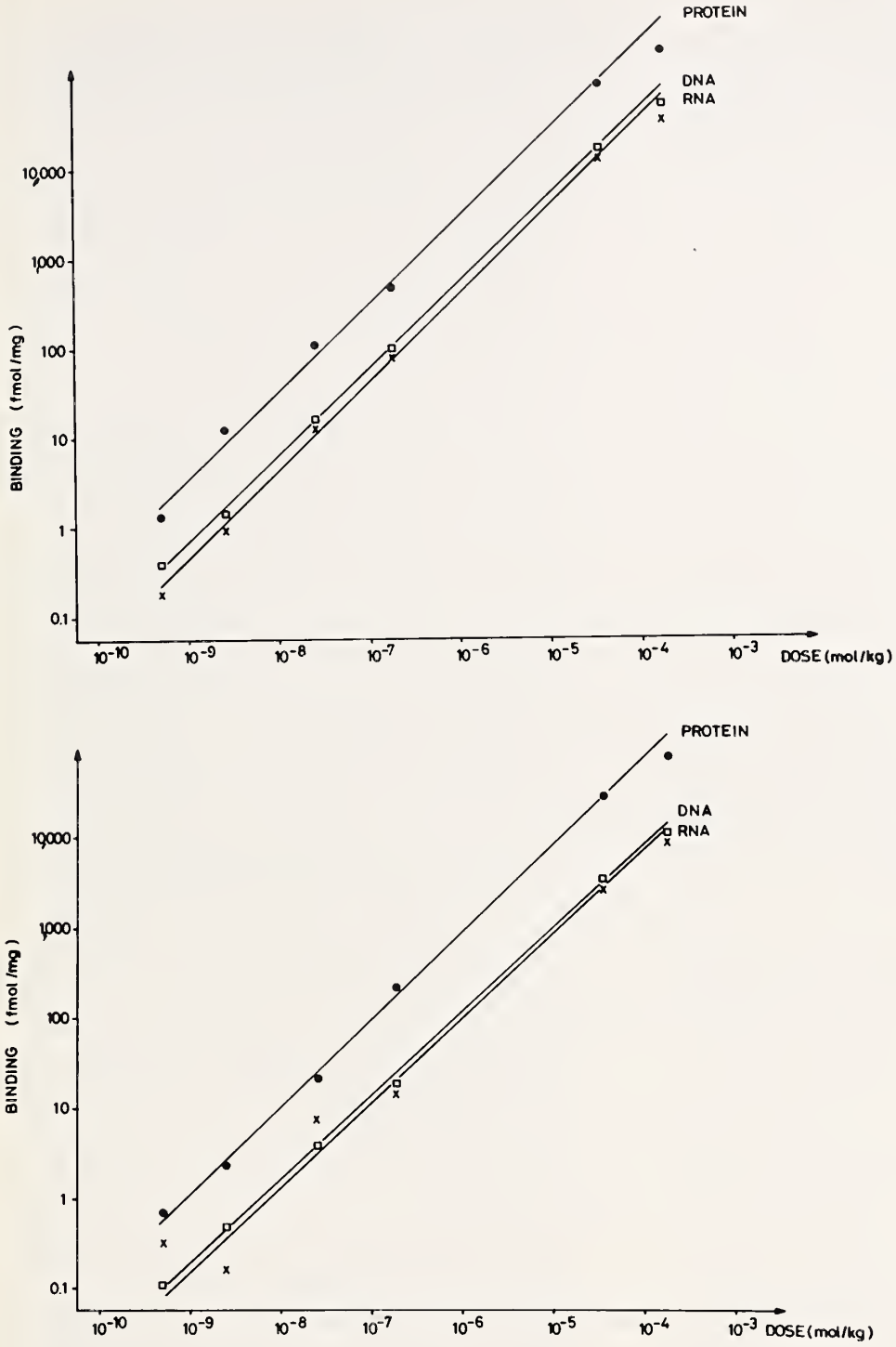
CONCLUSIONS

Aromatic amines, as exemplified by *trans*-DAS, are metabolically activated. Reactive metabolites, predominantly formed in the liver, produce primary biochemical lesions in all tissues. Chemical reactivity and pharmacokinetic parameters determine the extent of the reaction with cellular macromolecules. The reactions follow first-order kinetics, and no pharmacokinetically determined threshold exists. For *trans*-DAS, this has been demonstrated (text-fig. 4) down to a dose of 25 ng/animal (*23–25*).

[4] Baur H, Neumann H-G: Unpublished results.



TEXT-FIGURE 3.—Decrease with time of binding to tissue macromolecules. The [^{3}H]*trans*-DAS (10.5 μmol/kg body wt) was orally administered to 250-g female Wistar rats; groups of 4 animals were killed after 1, 14, and 28 days. Two livers were combined for processing. Protein, rRNA, and DNA were isolated as described in (*10*). Values represent an average of 2 determinations. Liver: Values for day 1 are apparently too high; therefore, values from previous experiments were included. Kidney: RNA and DNA were isolated from 4 organs with the use of hydroxyapatite columns after phenol extraction (Baur H: Unpublished results). One-day value: average from 2 determinations: 14- and 28-day values: 1 determination. Liver weight increased by 10% during the 28-day period. Blood proteins: For processing, *see* (*22*); values represent an average of 4 determinations.



TEXT-FIGURE 4.—Dose dependence of binding to cellular macromolecules. [^{3}H]*trans*-DAS was orally administered to female Wistar rats at various doses. After 24 hr, livers (*top*) and kidneys (*bottom*) from 2 animals for each dose were pooled, and proteins, rRNA, and DNA were isolated (*10, 24*). Deviations from linearity is not observed with low doses (the lowest dose was 25 ng/200 g body wt). Less than the proportionate fraction is bound with the highest dose.

TABLE 4.—*Carcinogenicity of* trans-*DAS in zymbalectomized rats* [a]

Treatment	No. of animals	Latent period, mo	Tumors	No. of animals with tumors
Control 1				
12 × 2 mg *trans*-DAS in 0.2 ml tricaprylin/200 g[c]	5	8–15 (12)	Ear duct	4[b]
Control 2				
Zymbalectomy + 12 × 0.2 ml tricaprylin	5	20		0
Zymbalectomy + 12×2mg *trans*-DAS in 0.2 ml tricaprylin/200 g[c]	5	9–12 (11)	Eyelid, lip[d]	5

[a] Zymbal's glands were removed surgically on both sides of female Wistar rats 2–3 wk before the compound was administered.

[b] One animal was lost during the experiment.

[c] The compound was injected ip twice a week in tricaprylin. Injections were discontinued after the sixth for 4 wk.

[d] The 2 eyelid tumors originated from Maibohm's glands, and the 3 lip tumors from sebaceous glands, all of which are similar to Zymbal's glands.

The susceptibility of a tissue is not directly related to its exposure to toxic metabolites. The proliferation rate should be considered as one contributing factor; other factors may be those which influence the promotion of initiated cells. In a most recent experiment, we showed that tumors developed elsewhere if the primary target tissue was removed (table 4; Marquardt P, Herrmann I, Neumann H-G: Unpublished observations).

REFERENCES

(*1*) HADDOW A, HARRIS RJ, KON GA, et al: The growth-inhibitory and carcinogenic properties of 4-aminostilbene derivatives. Philos Trans R Soc Lond [Ser A] 241:147–195, 1948

(*2*) SCHMÄHL D, MECKE R: Quantitative Untersuchung der carcinogen Wirksamkeit von 4-Amino-Stilbenen. Z Krebsforsch 61:230–239, 1956

(*3*) ANDERSEN RA, ENOMOTO M, MILLER EC, et al: Carcinogenesis and inhibition of the Walker 256 tumor in the rat by *trans*-4-acetylaminostilbene, its N-hydroxy metabolite, and related compounds. Cancer Res 24:128–143, 1964

(*4*) BALDWIN LW, SMITH WR: N-hydroxylation in aminostilbene carcinogenesis. Br J Cancer 19:433–443, 1965

(*5*) NEUMANN H-G, METZLER M, BRACHMANN I, et al: Zur Bedeutung chemisch-biologischer Wechselwirkungen für die toxische und krebserzeugende Wirkung aromatischer Amine. I. Krebserzeugende Wirksamkeit einiger 4-Aminostilben- und 4-Aminobibenzyl-Verbindungen. Z Krebsforsch 74:200–206, 1970

(*6*) NEUMANN H-G: Über die Darstellung von carcinogenen Aminen aus hydrierbaren Vorstufen und die Stabilität der eingeführten Wasserstoff-Atome gegen Austauschreaktionen. Hoppe Seyler's Z Physiol Chem 348:313–318, 1967

(*7*) RJOSK HK, NEUMANN H-G: Zur Bedeutung chemisch-biologischer Wechselwirkungen für die toxische und krebserzeugende Wirkung aromatischer Amine. II. Verteilung der Radioaktivität nach Applikation des Tritium-markierten Carcinogens *trans*-4-Dimethylaminostilben und der beiden unwirksamen Vergleichssubstanzen *cis*-4-Dimethylaminostilben und 4-Dimethylaminobibenzyl in der Ratte. Z Krebsforsch 75:209–220, 1971

(*8*) METZLER M, NEUMANN H-G: Zur Bedeutung chemisch-biologischer Wechselwirkungen für die toxische und krebserzeugende Wirkung aromatischer Amine. IV. Stoffwechselmuster von *trans*-4-Dimethylaminostilben, *cis*-4-Dimethylaminostilben und 4-Dimethylaminobibenzyl in Leber, Niere und den Ausscheidungsprodukten der Ratte. Z Krebsforsch 76:16–39, 1971

(*9*) _____: Epoxidation of the stilbene double bond, a major pathway in aminostilbene metabolism. Xenobiotica 7:117–133, 1977

(*10*) GAUGLER BJ, NEUMANN H-G: The binding of metabolites formed from aminostilbene derivatives to nucleic acids in the liver of rats. Chem Biol Interact 24:355–372, 1979

(*11*) SCRIBNER NK, SCRIBNER JD, SMITH PL, et al: Reactions of the carcinogen *N*-acetoxy-4-acetamidostilbene with nucleosides. Chem Biol Interact 26:27–46, 1979

(*12*) SCRIBNER NK, SCRIBNER JD: Reactions of the carcinogen *N*-acetoxy-4-acetamidostilbene with polynucleotides in vitro. Chem Biol Interact 26:47–55, 1979

(*13*) GAUGLER BJ, NEUMANN H-G, SCRIBNER NK, et al: Identification of some products from the reaction of *trans*-4-aminostilbene metabolites and nucleic acids in vivo. Chem Biol Interact 27:335–342, 1979

(*14*) DURSTON WE, AMES BN: A simple method for the detection of mutagens in urine: Studies with the carcinogen 2-acetylaminofluorene. Proc Natl Acad Sci USA 71:737–741, 1974

(*15*) GLATT HR, OESCH F, NEUMANN H-G: Factors responsible for the metabolic formation and inactivation of bacterial mutagens from *trans*-4-acetylaminostilbene. Mutat Res 73:237–250, 1980

(*16*) SCHENK J, NEUMANN H-G: The role of lipophilicity for the exposure of extrahepatic tissues in the rat after oral administration of aminostilbene derivatives. Xenobiotica 10:675–688, 1980

(*17*) ODASHIMA S: Development of liver cancer in the rat by 4-dimethylaminostilbene feeding following initial 4-dimethylaminoazobenzene feeding. I. Examination of the rat fed initially with DAB for more than one month. Gan 51 (Suppl):148, 1960

(*18*) TAKAYAMA S: Skin tumors in ACI/N rats induced by 3-methylcholanthrene and 4-dimethylaminostilbene. Gan 61:367, 1970

(*19*) SCHRAMM T, BIELKA H, GRAFFI A: Geschwulsterzeugung durch chemische Substanzen. XXIII. Stilbene. *In* Handbook of Pharmacology, vol XVI, Part 12. Berlin: Springer-Verlag, 1966, pp 167–176

(*20*) MARQUARDT P, NEUMANN H-G, ROMEN W: Tissue-specific, acute toxic effects of the carcinogen *trans*-4-dimethylaminostilbene. J Environ Pathol Toxicol. In press

(*21*) NEUMANN H-G: Pharmacokinetic parameters influencing tissue specificity in chemical carcinogenesis. Arch Toxicol 41 (Suppl 2):229–238, 1979

(*22*) WIELAND E, NEUMANN H-G: Methemoglobin formation and binding to blood constituents as indicator for the formation, availability and reactivity of activated metabolites from *trans*-4-aminostilbene and related aromatic amines. Arch Toxicol 40:17–35, 1978

(*23*) NEUMANN H-G, GAUGLER BJ, TAUPP W: The metabolic activation of *trans*-4-dimethylaminostilbene after oral ad-

ministration of doses ranging from 0.025 to 250 μmol/kg. *In* Proceedings of the First International Congress on Toxicology (Plaa D, ed). New York: Academic Press, 1978, pp 177–190

(*24*) NEUMANN H-G, BAUR H, WIRSING R: Dose–response relationships in the primary lesion of strong electrophilic carcinogens. Arch Toxicol 42 (Suppl 3):69–77, 1980

(*25*) NEUMANN H-G: Biochemical effects and early lesions in regard to dose-response studies. Oncology 37:255–258, 1980

Determinants of Nucleic Acid Adduct Formation[1, 2]

John D. Scribner[3]

ABSTRACT—Although *N*-aryl- or *N*-acetyl-*N*-arylnitrenium ions are considered the reactive species in the attack of many aromatic amines or amides on biologic macromolecules, a coherent picture explaining the variety of reactions of the ultimate carcinogens from which they are derived has not been established. Self-consistent field–molecular orbital calculations were used to attempt to understand the reactions of 3 ultimate carcinogens in the aromatic amine class. The MINDO/3 calculations for the cations resulting from loss of acetate from 3-acetoxyxanthine, *N*-acetoxy-2-fluorenylacetamide (*N*-AcO-2-FAA) and *N*-acetoxy-4-acetamidostilbene show that these cations are unlikely to exist in a triplet state, contrary to experimental data which could be interpreted otherwise. Iterative extended Hückel theory calculations for these cations show that the reactions which suggested a triplet state may be explained on the basis of hard/soft-acid/base theory. Electrostatic potential maps combined with frontier orbital theory suggest a coherent explanation for the variety of reactions of *N*-AcO-2-FAA. The use of the polyelectronic perturbation theory has previously been shown to predict correctly the major product of reaction between guanosine and 5 ultimate carcinogens. I have shown here that the unmodified theory (using Hückel molecular orbital theory) further predicts the major product of reaction between DNA and *N*-hydroxy-2-naphthylamine, but that it predicts secondary products incorrectly.—Natl Cancer Inst Monogr 58: 173–181, 1981.

The history of the nitrenium ion in carcinogenesis research began in 1965 with two short papers. While working in the Millers' laboratory, I showed that a significant portion of carcinogenic azo dye bound to rat liver protein is in a form which releases a sulfide on cold alkaline hydrolysis (*1*). This 3-methylmercapto-*N*-methyl-4-aminoazobenzene was obviously derived from methionine and probably from an N-oxidized azo dye, but we could not imagine a mechanism to accomplish that. Within a month, Kriek (*2*) reported that treating RNA or DNA with *N*-hydroxy-2-fluorenamine in dilute acid resulted in binding the fluorenamine chromophore to the nucleic acid, particularly to guanine. He also proprosed, but could not prove at the time, that the amine nitrogen was attached to the C-8 of guanine. However, he had no indication that such a reaction took place in vivo. Thus we had an in vivo event without a mechanism, and Dr. Kriek had a mechanism without proved significance. It was our good fortune in the Miller laboratory to relate these findings through the use of the esters *N*-benzoyloxy-MAB and *N*-AcO-2-FAA. Both reacted with methionine at pH 7.4 to give methylmercapto adducts of MAB and 2-FAA, respectively, which were identical with adducts found in livers of rats given *N,N*-dimethyl-4-aminoazobenzene or 2-FAA (*1, 3–5*). A similar story regarding nucleic acid adducts has also been developed (*6–8*). Most importantly, we could show then that *N*-OH-2-FAA did not undergo this reaction, and Dr. Kadlubar (*9, 10*) has shown more recently that *N*-OH-MAB is similarly inactive at neutrality. Thus it appeared that the ester function was necessary as a leaving group. The metabolic routes leading to such esters in vivo have already been discussed.

My concern has been the nature of the reaction of the ester. It could easily be shown that the rate of decomposition of esters was dependent on the concentration of organic solvent in the medium (*11*), which suggested strongly that ions were being formed from nonionic starting material. On this basis, we proposed that the active intermediate in the reactions of *N*-acetoxy-*N*-arylacetamides was an *N*-aryl-*N*-acetylnitrenium ion. The MO calculations based on this assumption gave satisfying agreement between predicted and observed relative reaction rates for 6 *N*-acetoxy-*N*-arylacetamides (*11*). The nitrenium ion has been used to explain the formation of *p*-aminophenol from phenylhydroxylamine by sulfuric acid, or of *p*-chloroaniline by hydrochloric acid (*12*). Dr. Kriek noted this when he published his initial finding (*2*). Our results were an indirect demonstration that Dr. Kriek was probably right regarding in vivo significance. Direct demonstrations have since come from Dr. King, Dr. Kriek, and others.

A problem that surfaced early and led directly to the title of this paper was the observation that the rate of reaction of *N*-acetoxy-*N*-arylacetamides was unrelated to the yield of adduct with nucleoside or nucleic acid. One could ask: Are nitrenium ions involved in all chemical attack by aromatic amines or amides on nucleic acids in vivo? If so, what are the features of the individual ions which permit greater or lesser reaction with nucleoside or nucleic acid, and which select for reaction at one base in preference to another? Such questions refer to the experimental results

Abbreviations: *N*-AcO-2-FAA = *N*-acetoxy-2-fluorenylacetamide; MAB = *N*-methyl-4-aminoazobenzene; *N*-OH-2-FAA = *N*-hydroxy-2-FAA; MO = molecular orbital; DPPH = diphenylpicrylhydrazyl; *N*-AcO-4-AAS = *N*-AcO-4-acetamidostilbene; IEHT = iterative extended Hückel theory; FAA^+ = acetylfluorenylnitrenium ion; AAS^+ = acetylstilbenylnitrenium ion; Å = angstrom.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported by Public Health Service grant CA23712 and contract CP85628 (with the Division of Cancer Cause and Prevention) from the National Cancer Institute.

[3] Pacific Northwest Research Foundation, 1124 Columbia Street, Seattle, Washington 98104.

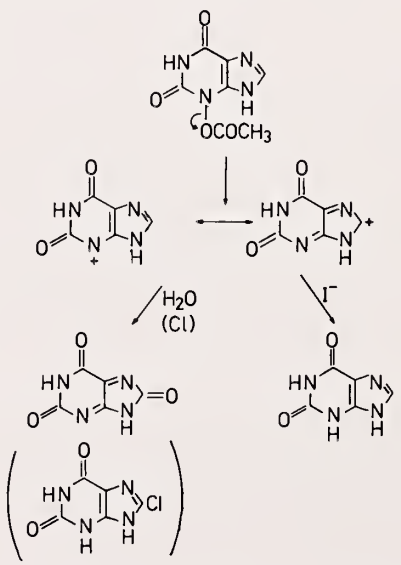
already discussed. One could also ask: Do the different kinds of attack expected or observed result in different levels or types of mutagenesis and/or carcinogenesis? This is a question which leads to papers to be presented in this Symposium. Let us first look at properties of these nitrenium ions in isolation.

One which consumed some effort on our part and that of others is the question: Is the nitrenium ion a radical? We (*13, 14*) reported evidence that the *N*-acetyl-*N*-fluorenylnitrenium ion is a triplet, i.e., that the positive ion formed by loss of an acetate or sulfate ion immediately changes to a new electron rearrangement in which there are 2 unpaired electrons. The evidence was of two kinds: 1) *N*-AcO-2-FAA decolorized the stable free radical DPPH in aqueous medium but not in 95% ethanol, and the rate of this decomposition was equal to the rate of decomposition in the absence of DPPH (*13*). However, *N*-AcO-4-AAS, which decomposes at only a slightly lower rate than *N*-AcO-2-FAA, had no effect on DPPH. 2) A small amount of FAA is formed in solvolysis of *N*-AcO-2-FAA, as first reported by Dr. Lotlikar (*15*). In addition, we (*14*) found that ascorbic acid tremendously increases the amount of 2-FAA formed in the reaction between guanosine and *N*-AcO-2-FAA and correspondingly reduces the amount of guanosine-2-FAA adduct. Again, the reaction of *N*-AcO-4-AAS was insensitive to like medium changes. No AAS was formed, and ascorbic acid appeared to be without effect. We interpreted these results as evidence that the *N*-acetyl-*N*-fluorenylnitrenium ion is a triplet, but that the *N*-acetyl-*N*-stilbenylnitrenium ion is not. However, studies on 3-acetoxyxanthine by Templeton and Parham (*16*) have shown that this compound reacts as an electrophilic nitrenium ion with hard nucleophilies but is simply reduced to xanthine by soft nucleophiles (text-fig. 1). One could attempt to unify these findings by scrapping the triplet idea or by insisting that the xanthine cation is also a triplet ion. Again, MO calculations offer one way out of the quandary. They can answer two questions: What are the relative energies of the two possible electronic states for the nitrenium ions? Which electronic state better explains the known sites of reaction of the ions?

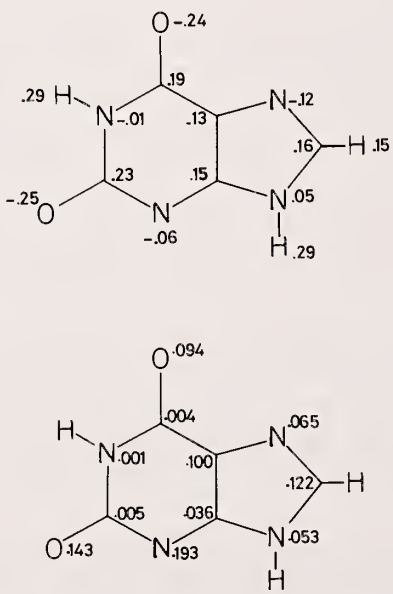
The MINDO/3 semiempirical self-consistent field–MO method (*17*) was used in calculations of the heats of formation of the different ions. For the xanthine singlet and triplet cations, the program was allowed to optimize all bond lengths, bond angles, and twist angles to obtain minimum energies for both ions. The result was that the triplet was less stable than the singlet by 11.8 kcal/mol. If electron distributions are calculated by the IEHT (*18*), which is better than MINDO/3 for this purpose, we find that the charge is higher on C-8 (text-fig. 2), which is where water, methionine, and the chloride ion attack, than on N-3. However, frontier orbital reactivity is greater at N-3. Hence softer species (*19*), such as iodide ion or thiourea, would be expected to react at this position. If iodide did attack N-3, this action might be followed by homolytic cleavage to give atomic iodine and a doublet xanthine radical, which could abstract hydrogen from the solvent to be reduced.

Application of this approach to the FAA^+ and the AAS^+ shows that for both the triplet is 20.8 kcal/mol less stable than is the singlet. Because AAS^+ shows no properties of a triplet, it is unlikely that either is a triplet. Examination of the IEHT electron distribution in singlet FAA^+ shows the greatest charge to be on the carbonyl carbon (text-fig. 3), with the charges on C-1 and C-3 only slightly greater than neutrality. The charge at nitrogen is negative. Frontier orbital reactivity is clearly greatest at the nitrogen atom. Although this result explains the attack on the little-charged C-8 of guanine, it does not explain the reaction of water with C-4 or of methionine or Cl^- at C-3 and C-1.

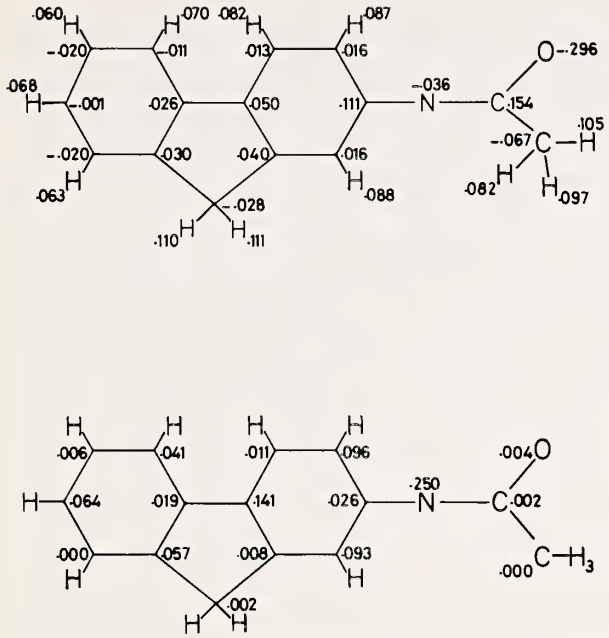
Another way to view these ions is through the use of



TEXT-FIGURE 1.—Reactions of 3-acetoxyxanthine in water (*16*).



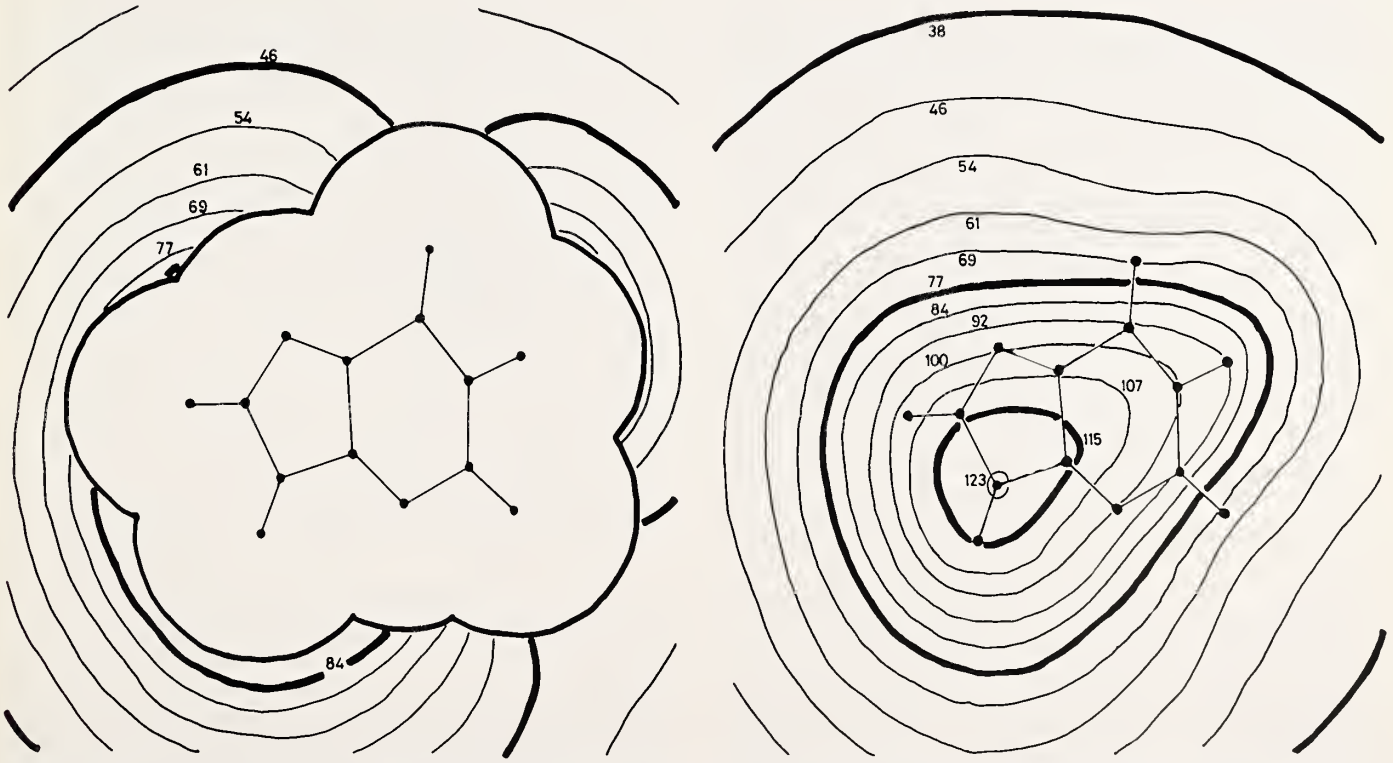
TEXT-FIGURE 2.—Electron distribution in xanthinium ion. *Upper:* Mulliken charges; *lower:* frontier orbital coefficients (p_z atomic orbitals in the lowest unoccupied MO).



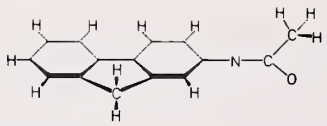
TEXT-FIGURE 3.—Electron distribution in *N*-2-fluorenyl-*N*-acetylnitrenium ion. *Upper,* Mulliken charges; *lower,* frontier orbital coefficients.

electrostatic potential maps. Atomic charges are artificial concepts, whereas the electrostatic potential at any point in space is conceptually a real property due to the combined influences of the fields from all of the nuclei and electrons in a molecule. Ideally, a map of such potentials at any arbitrary number of points could be obtained by integration over all of the nuclei in the molecule and over all of the wave functions for the electrons, the latter obtained from ab initio calculations (*20*). One can make a first approximation to this procedure by using wave functions obtained from a semiempirical method which gives a good approximation to ab initio wave functions (*21*). We are still developing such a method on our computer, so I am presenting here some suggestive results obtained from a second approximation, e.g., summing the potentials due to the calculated partial charges for the individual atoms. We found that, for the nucleic acid bases, the maps obtained in this manner for points at least 2 Å from the nearest nucleus are remarkably similar to maps calculated by the most rigorous methods. We calculated the charges by using IEHT. For the xanthine cation, we obtained the pictures shown in text-figure 4. One map is drawn simply 2 Å above the aromatic plane. Here we see that the positive potential directly above C-8 is about 13 kcal/mol greater than that above N-3. In the ring plane, with the 2-Å periphery plotted, we see that the difference is not as pronounced but also that a high potential is spread over a wider area near the imidazole ring.

The FAA^+ picture is more interesting because of the geometry of the cation itself (text-fig. 5). According to MINDO/3 calculations (contrary to pictures which I presented earlier), the acetyl group is neither coplanar with the ring nor at a 120° angle to C-2. Instead, C-2, N, and the



TEXT-FIGURE 4.—Electrostatic potential maps for xanthinium ion. *Left:* in molecular plane; *right:* 2 Å from molecular plane. Units are kilocalories/mole equivalents of calculated electron volts.



TEXT-FIGURE 5.—Geometry of the *N*-2-fluorenyl-*N*-acetylnitrenium ion.

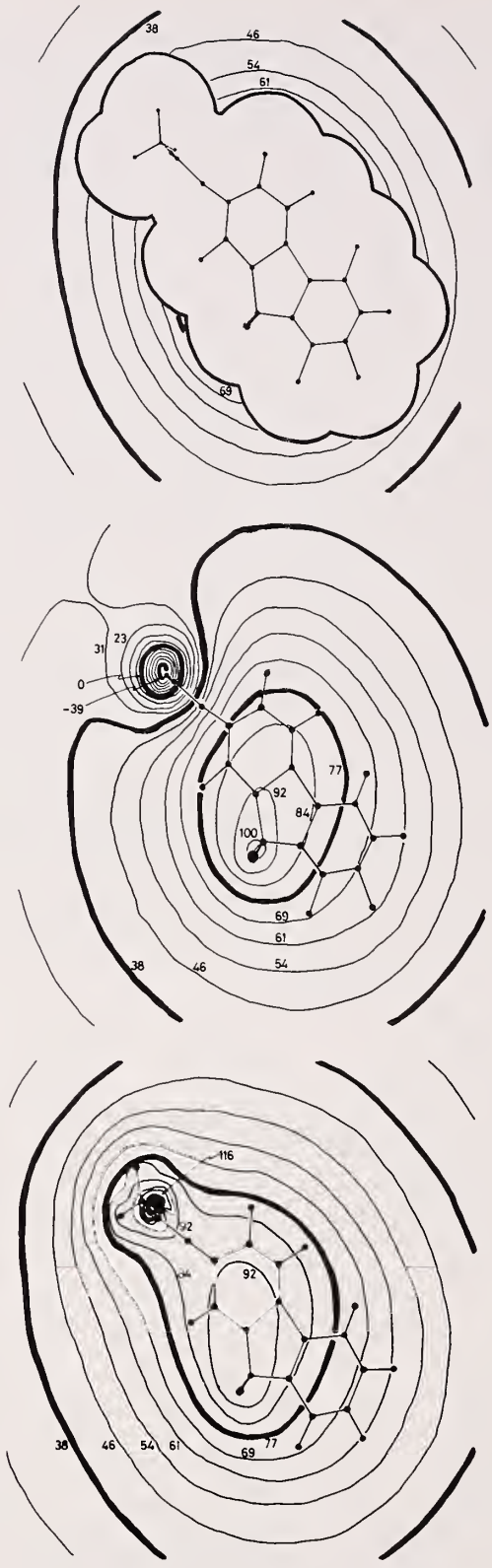
carbonyl carbon are essentially in a straight line. The carbonyl oxygen is twisted out of the plane by about 90°, and the methyl group is on the opposite side of the ring.

In the potential maps (text-fig. 6), we find nothing exceptional in the ring plane. The highest potential at the 2-Å periphery is near the CH_2 bridge, with the next highest near the 1, 3, and 4 positions. On the oxygen side of the molecule and 2 Å from the plane, the highest potential is again near the CH_2 group, with that at positions 1 and 4 about equal and lower and that at position 3 another 2–3 kcal less. As one might expect, there is actually a negative potential above the oxygen, although the relative value is questionable due to the proximity of the oxygen nucleus.

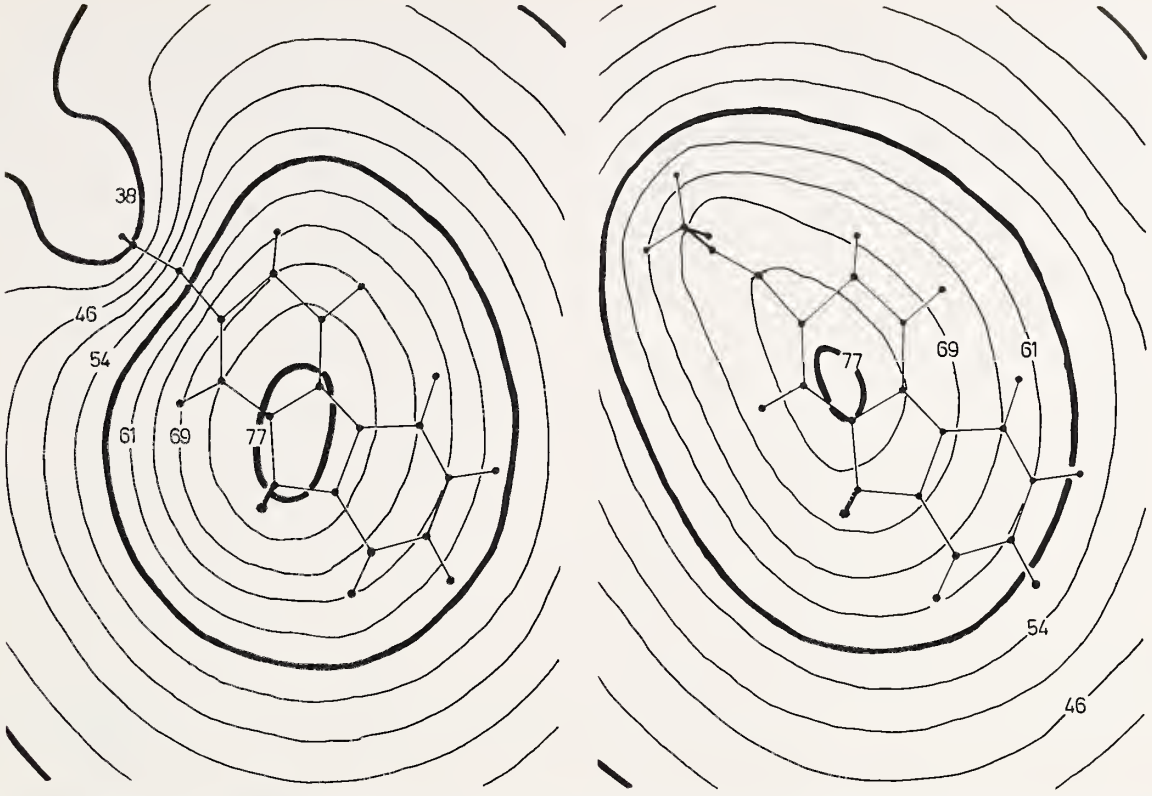
A high positive potential on the opposite side of the plane is due to a hydrogen nucleus in the map plane. To circumvent the problem of out-of-plane nuclei, we prepared new maps farther from the ring plane (text-fig. 7). On the carbonyl side of the ring and at this greater distance, the potential at C-4 is 20 kcal higher than at N, 5 kcal higher than at C-3, and about 2 kcal higher than at C-1. Thus at this distance, preference for charge-controlled attack at C-4 is clear. On the methyl side, the potential is uniform, with C-1 about 3 kcal higher than either C-3 or C-4.

From these text-figures, we can conclude that the slight negative charge calculated to be on N is not seen by an approaching nucleophile. Consequently, soft nucleophiles (*19*) can be expected to attack N preferentially, with its higher frontier orbital reactivity. Harder nucleophiles can be expected to seek out the carbonyl carbon or ring carbons 1, 3, or 4. Because of probable reversibility at the carbonyl group and some steric hindrance at C-1, let us again compare only 3 and 4. These are comparably reactive in the ring plane and from the methyl side, whereas 4 is favored from the oxygen side by as much as 7 kcal/mol. Thus water, a hard nucleophile (*19*), can be expected to attack primarily at C-4, as has been observed (*22*). Note also that the even higher potential near position 4a supports the mechanism previously proposed (*22*).

Although charged, chloride ion is a softer species than water (*19*), presumably because its charge is spherically diffused over larger orbitals than those in water. Thus its reactivity may be governed more by overlap with vacant orbitals. The N–Cl overlap is not as favorable as C–Cl overlap, leaving C-3, which has been observed (*22*) to be the major reaction site of *N*-AcO-2-FAA with ammonium chloride (solution). Uncharged methionine would be expected to behave similarly. If water, chloride, and methionine attack C-8 in xanthine, why does only water attack C-4 in FAA^+ ? In the xanthine cation, the only reasonable alternatives are heteroatoms, with reduced overlap toward heteroatom nucleophiles. In FAA^+, other carbon atoms are



TEXT-FIGURE 6.—Electrostatic potential maps for *N*-2-fluorenyl-*N*-acetylnitrenium ion. *Top:* in molecular plane; *middle:* 2 Å from molecular plane, carbonyl side; *bottom:* 2 Å from molecular plane, methyl side. Units are kilocalories/mole.

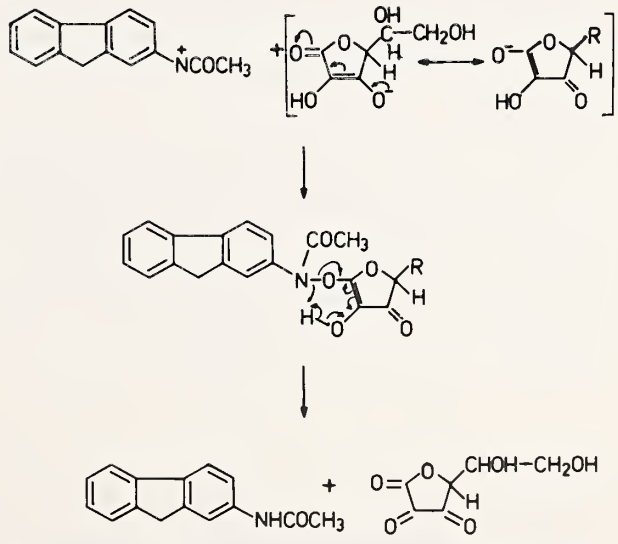


TEXT-FIGURE 7.—Electrostatic potential maps for *N*-2-fluorenyl-*N*-acetylnitrenium ion. *Left:* 3.44 Å from molecular plane, carbonyl side; *right:* 3.99 Å from molecular plane, methyl side. Units are kilocalories/mole.

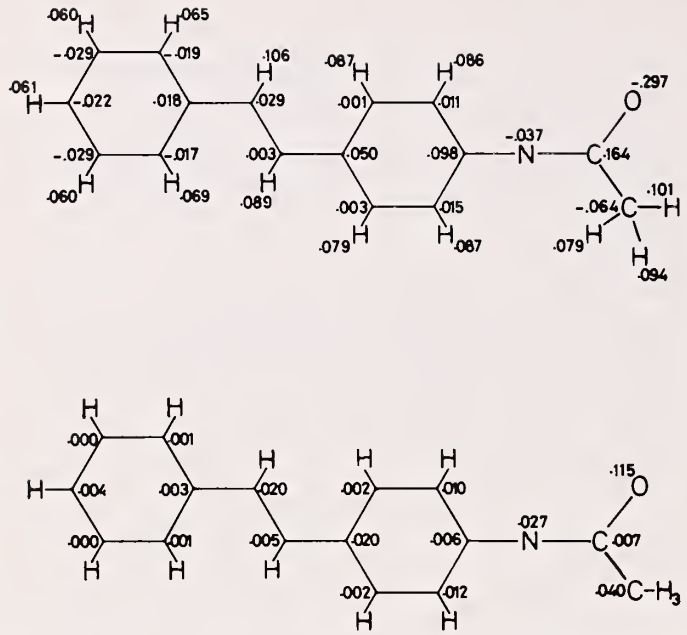
available. In addition to the lower overlap between heteroatoms, the frontier orbital density on N-3 in the xanthine cation is only 50% higher than on C-8. In FAA^+, the frontier orbital density on C-3 is eight times higher than on C-4. Also, if water also preferentially attacks C-3, H-bonding between the hydroxyl and acetyl groups may stabilize the quinoid intermediate sufficiently to allow addition of a second molecule of water to C-4 similarly to that proposed earlier (*22*). Deprotonation of a chloride or methionine adduct would probably be fast enough to prevent such a second addition. (I thank Dr. George Ford for this suggestion.)

Thus at the moment, we have what seems to be a satisfactory qualitative explanation of the known chemistry of *N*-AcO-2-FAA. We may now ask how ascorbic acid reduces FAA^+. Because of the extensive delocalization in the ascorbate ion, this ion may be soft enough to attack the nitrogen in the FAA^+, a reaction that could be followed by homolytic cleavage and reduction of the FAA radical to FAA (text-fig. 8).

The AAS^+ picture is different (text-fig. 9). Now there is little difference in frontier orbital densities between N and the most reactive carbon, whereas the difference in potentials has increased (text-figs. 10, 11). As a result, potential plays the dominant role in governing reaction site, and one would expect both hard and soft nucleophiles to attack the double-bond region. In fact, water, methionine, and nucleosides attack here (*23–25*). The low frontier orbital coefficients for AAS^+ generally, in comparison with FAA^+, also explain why yields of nucleoside adducts are low with AAS^+ but high with FAA^+ because the soft nucleosides can



TEXT-FIGURE 8.—Hypothetical reaction between ascorbate ion and *N*-2-fluorenyl-*N*-acetylnitrenium ion. Electron redistribution to cleave the intermediate could be heterolytic but would require formal hydride transfer.

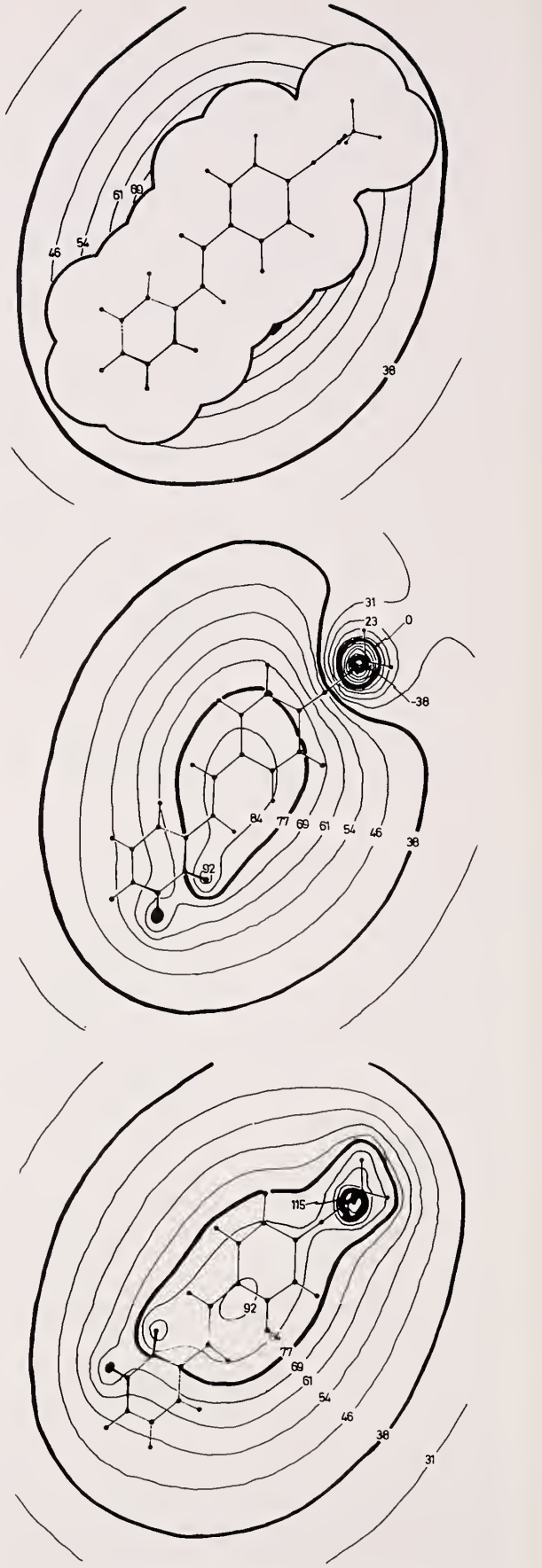


TEXT-FIGURE 9.—Electron distribution in *N*-4-stilbenyl-*N*-acetylnitrenium ion. *Upper,* Mulliken charges; *lower,* frontier orbital coefficients.

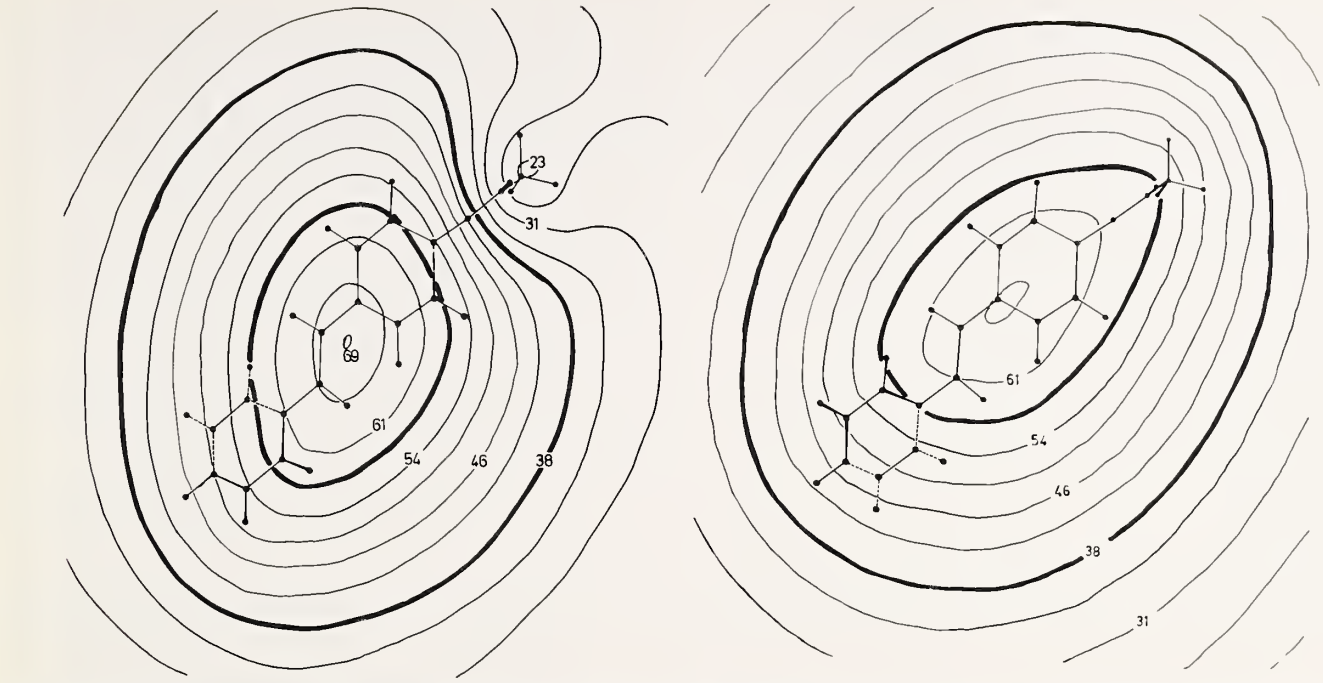
compete with the harder water for FAA^+ better than they can for AAS^+.

Inasmuch as we know that different nitrenium ions have different selectivities for nucleic acid bases (*25–28*), we can ask how these are governed. From the above, we see that the same principles apply to attack at the various positions on the nucleic acid bases modified by steric requirements. We (*29*) have already published the beginnings of a predictive method based on Klopman's approach (table 1). This method, without any modification, predicted the major product from Dr. Kadlubar's (*30*) studies with DNA and *N*-hydroxy-2-naphthylamine (table 2). It did not do so well with secondary adducts and will not be completely successful until it can do that and also predict the extent of competition by water.

The significance of being able to make such predictions is demonstrated by the observation that different types of genetic damage are associated with different aryl groups on aromatic amines and amides (tables 3, 4). By predicting site and degree of reaction, we may also predict level and type of mutagenicity (*31*). How closely these are related to cancer, we do not know, but detailed knowledge of the chemistry of the initial events in these processes will remove a major blind spot from future work.



TEXT-FIGURE 10.—Electrostatic potential maps for *N*-4-stilbenyl-*N*-acetylnitrenium ion. *Top:* in plane of aniline ring; *middle:* 2 Å from plane of aniline ring, carbonyl side; *bottom:* 2Å from plane of aniline ring, methyl side. Units are kilocalories/mole.



TEXT-FIGURE 11.—Electrostatic potential maps for *N*-4-stilbenyl-*N*-acetylnitrenium ion. *Left:* 3.44 A from plane of aniline ring, carbonyl side; *right* 3.99 Å from plane of aniline ring, methyl side. Units are kilocalories/mole.

TABLE 1.—*Reactions of arylnitrenium ions with guanosine*[a]

Position on guanosine	*N*-acetyl-*N*-2-fluorenyl-nitrenium ion		*N*-acetyl-*N*-4-xenylnitrenium ion		*N*-acetyl-*N*-2-phenanthryl-nitrenium ion		*N*-1-naphthylnitrenium ion	
	3	N	3	N	1	N	4	N
1	0.14	0.02	0.14	0.2	0.10	0.02	0.18	0.06
3	0.03	0.03	0.03	0.02	0.05	0.03	0.08	0.02
7	0.14	0.07	0.14	0.06	0.21	0.07	0.16	0.10
8	0.10	0.26	0.20	0.30	0.17	0.34	0.11	0.22
N^2	0.19	0.08	0.18	0.08	0.27	0.09	0.22	0.20
O^6	0.17	0.04	0.18	0.04	0.15	0.05	0.19	0.28

[a] Sign conventions were chosen so that a higher value indicates a more favorable reaction. The known predominant reactions of precursor compounds are indicated by *underlining*.

TABLE 2.—*Reaction of 2-naphthylhydroxylamine* (N-*2-naphthylnitrenium ion*) *with nucleosides* [a]

Position on nitrenium ion	Position on nucleoside base	Guanosine	Position on nucleoside base	Adenosine	Position on nucleoside base	Cytidine	Position on nucleoside base	Thymidine
C-1	1	0.12	1	0.09	3	0.11	3	0.11
	3	0.04	3	0.03	5	0.24	O^2	0.02
	7	0.10	7	0.10	O^2	0.04	O^4	0.21
	8	0.24	8	0.20	N^4	0.24		
	N^2	0.25	N^6	0.26				
	O^6	0.13						
N	1	0.13	1	0.13	3	0.15	3	0.14
	3	0.06	3	0.07	5	0.25	O^2	0.26
	7	0.13	7	0.13	O^2	0.32	O^4	0.29
	8	0.40	8	0.31	N^4	0.25		
	N^2	0.27	N^6	0.27				
	O^6	0.34						

[a] Calculations were done as in (*27*). Compare with table 1 and with (*30*).

TABLE 3.—*Mutagenesis by* N-*acetoxy*-N-*arylacetamides and nitroarenes in* Salmonella typhimurium[a]

Aryl substituent	Strain:			
	TA98	TA1538	TA100	TA1535
N-Acetoxy-*N*-arylacetamides				
2-Fluorene	8.0	4.3	0.3	0.1
4-Biphenyl	0.3	0.1	0.2	0.1
2-Phenanthrene	14.9	13.2	3.7	0.3
4-Stilbene	2.8	0.4	6.8	0.0
1-Naphthalene	0.2	0.2	0.4	0.3
2-Naphthalene	0.5	0.1	4.6	2.8
Nitroarenes				
2-Fluorene	51.1	65.0	7.9	0.0
4-Biphenyl	1.4	0.9	4.2	0.0
2-Phenanthrene	128	145	62.4	2.7
4-Stilbene	2.3	1.0	11.7	0.0
1-Naphthalene	0.2	0.1	0.8	0.0
2-Naphthalene	0.2	0.4	4.8	3.7
2-Anthracene	892	839	1,133	794

[a] No activating system was added. Values are expressed as revertants/nanomoles at optimum dose.

TABLE 4.—*Mutagenesis by arylamines and* N-*arylacetamides in* Salmonella typhimurium[a]

Aryl substituent	Strain:			
	TA98	TA1538	TA100	TA1535
Arylamines				
2-Fluorene	85.4	92.8	42.3	0.4
4-Biphenyl	6.8	4.7	11.8	0.2
2-Phenanthrene	726	566	102	15.9
4-Stilbene	2.9	2.4	12.6	0.0
1-Naphthalene	0.2	0.1	5.1	2.9
2-Naphthalene	1.6	0.6	10.7	14.7
2-Anthracene	77	95	77	13.8
N-arylacetamides				
2-Fluorene	16.1	26.8	7.0	0.0
4-Biphenyl	1.0	0.3	1.8	0.1
2-Phenanthrene	14.1	32.7	17.0	2.7
4-Stilbene	2.5	2.2	13.6	0.5
1-Naphthalene	0.0	0.0	0.0	0.1
2-Naphthalene	0.0	0.0	0.0	0.0
2-Anthracene	92	109	79	79

[a] Values indicate revertants/nanomoles at optimum concentrations of mutagen and rat liver S-9.

REFERENCES

(*1*) SCRIBNER JD, MILLER JA, MILLER EC: 3-Methylmercapto-*N*-methyl-4-aminoazobenzene: An alkaline-degradation product of a labile protein-bound dye in the livers of rats fed *N,N*-dimethyl-4-aminoazobenzene. Biochem Biophys Res Commun 20:560–565, 1965

(*2*) KRIEK E: On the interaction of *N*-2-fluorenylhydroxylamine with nucleic acids in vitro. Biochem Biophys Res Commun 20:793–799, 1965

(*3*) LOTLIKAR PD, SCRIBNER JD, MILLER JA, et al: Reaction of esters of aromatic *N*-hydroxy amines and amides with methionine in vitro: A model for in vivo binding of amine carcinogens to protein. Life Sci 5:1263–1269, 1966

(*4*) DEBAUN JR, MILLER EC, MILLER JA: *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methionin-*S*-yl) binding in rat liver. Cancer Res 30:577–595, 1970

(*5*) POIRIER LA, MILLER JA, MILLER EC, et al: *N*-Benzoyloxy-*N*-methyl-4-aminoazobenzene: Its carcinogenic activity in the rat and its reactions with proteins and nucleic acids and their constituents in vitro. Cancer Res 27:1600–1613, 1967

(*6*) KRIEK E, MILLER JA, JUHL U, et al: 8-(*N*-2-Fluorenylacetamido)-guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide in neutral solution. Biochemistry 6:177–182, 1967

(*7*) LIN J-K, SCHMALL B, SHARPE ID, et al: N-Substitution of carbon 8 in guanosine and deoxyguanosine by the carcinogen *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene in vitro. Cancer Res 35:832–843, 1975

(*8*) LIN J-K, MILLER JA, MILLER EC: Structures of hepatic nucleic acid-bound dyes in rats given the carcinogen *N*-methyl-4-aminoazobenzene. Cancer Res 35:844–850, 1975

(*9*) KADLUBAR FF, MILLER JA, MILLER EC: Microsomal N-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Cancer Res 36: 1196–1206, 1976

(*10*) _____: Hepatic metabolism of *N*-hydroxy-*N*-methyl-4-aminoazobenzene and other N-hydroxyarylamines to reactive sulfuric acid esters. Cancer Res 36:2350–2359, 1976

(*11*) SCRIBNER JD, MILLER JA, MILLER EC: Nucleophilic substitution on carcinogenic *N*-acetoxy-*N*-arylacetamides. Cancer Res 30:1570–1579, 1970

(*12*) HELLER HE, HUGHES ED, INGOLD CK: A new view of the arylhydroxylamine rearrangement. Nature 168:909–910, 1951

(*13*) SCRIBNER JD, NAIMY NK: Reactions of esters of *N*-hydroxy-2-acetamidophenanthrene with cellular nucleophiles and the formation of free radicals upon decomposition of *N*-acetoxy-*N*-arylacetamides. Cancer Res 33:1159–1164, 1973

(*14*) SCRIBNER JD, NAIMY NK: Destruction of triplet nitrenium ion by ascorbic acid. Experientia 31:470–471, 1975

(*15*) LOTLIKAR PD, LUHA L: Acylation of carcinogenic hydroxamic acids by carbamoyl phosphate to form reactive esters. Biochem J 124:69–74, 1971

(*16*) TEMPLETON MA, PARHAM JC: Purine N-oxides. 65. On the mechanisms of the reactions of 3-acetoxyxanthine. J Org Chem 43:544–550, 1978

(*17*) BINGHAM RC, DEWAR MJ, LO DH: Ground states of molecules. XXV. MINDO/3. An improved version of the MINDO semiempirical SCF-MO method. J Am Chem Soc 97:1285–1293, 1975

(*18*) SCHAFFER AM, GOUTERMAN M, DAVIDSON ER: Porphyrins. XXVIII. Extended Hückel calculations on metal phthalocyanines and tetrazaporphins. Theor Chim Acta 30:9–30, 1973

(*19*) KLOPMAN G: Chemical reactivity and the concept of charge- and frontier-controlled reactions. J Am Chem Soc 90: 223–234, 1968

(*20*) SCROCCO E, TOMASI J: The electrostatic molecular potential as a tool for the interpretation of molecular properties. Top Curr Chem 42:95–170, 1973

(*21*) PULLMAN A: Theoretical calculation of the reactivity of adenine and guanine toward alkylation. *In* Chemical Carcinogenesis (Ts'o PO, DiPaolo JA, eds). New York: Marcel Dekker, 1974, pp 375–378

(*22*) Scribner JD: Conversion of the carcinogen *N*-acetoxy-2-acetamidofluorene to 4-hydroxy-2-acetamidofluorene. J Am Chem Soc 99:7383–7384, 1977

(*23*) Miller EC, Butler BW, Fletcher TL, et al: Methylmercapto-4-acetylaminostilbenes as products of the reaction of *N*-acetoxy-4-acetylaminostilbene with methionine and as degradation products of liver protein from rats given *N*-hydroxy-4-acetylaminostilbene. Cancer Res 34:2232–2239, 1974

(*24*) Scribner JD: Solvolysis of the carcinogen *N*-acetoxy-*N*-(4-stilbenyl)acetamide: Solvent addition to an intermediate quinone imide methide. J Org Chem 41:3820–3824, 1976

(*25*) Scribner NK, Scribner JD, Smith DL, et al: Reactions of the carcinogen *N*-acetoxy-4-acetamidostilbene with nucleosides. Chem Biol Interact 26:27–46, 1979

(*26*) Kadlubar FF, Miller JA, Miller EC: Guanyl O^6-arylamination and O^6-arylation of DNA by the carcinogen *N*-hydroxy-1-naphthylamine. Cancer Res 38:3626–3638, 1978

(*27*) Kriek E: Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA in vivo. Cancer Res 32: 2042–2048, 1972

(*28*) Scribner JD, Naimy NK: Adducts between the carcinogen 2-acetamidophenanthrene and adenine and guanine of DNA. Cancer Res 35:1416–1421, 1975

(*29*) Scribner JD, Fisk SR: Reproduction of major reactions of aromatic carcinogens with guanosine, using HMO-based polyelectronic perturbation theory. Tetrahedron Lett 4759–4762, 1978

(*30*) Kadlubar FF, Unruh LE, Beland FA, et al: Formation of DNA adducts by the carcinogen *N*-hydroxy-2-naphthylamine. Natl Cancer Inst Monogr 58:143–152, 1981

(*31*) Scribner JD, Fisk SR, Scribner NK: Mechanisms of action of carcinogenic aromatic amines: An investigation using mutagenesis in bacteria. Chem Biol Interact 26: 11–25, 1979

Molecular Mechanism of Chemical Modification of Cellular Nucleic Acid Bases by 4-Hydroxyaminoquinoline 1-oxide[1]

Yutaka Kawazoe[2, 3]

ABSTRACT—The O-acyl derivative of 4-hydroxyaminoquinolone, the proposed ultimate metabolite in carcinogenesis by the parent compound, was proposed to undergo a nitrogen–oxygen heterolysis to produce three types of reaction intermediates: a nitrenium, a carbonium, and nitrene intermediates, which were considered to give N(4)-substituted 4-aminoquinoline 1-oxide (4-AQO), 3-substituted 4-AQO, and 4-AQO, respectively, in reactions with nucleophiles including nucleic acid bases. Chemical modification of cellular DNA by this carcinogen is discussed.—Natl Cancer Inst Monogr 58: 183–184, 1981.

Tada and Tada (*1, 2*) and Nagao and Sugimura (*3*) demonstrated that 4-HAQO, the proximate metabolite in 4-NQO carcinogenesis, converted to its aminoacyl conjugate in the presence of aminoacyl-tRNA synthetase. The latter metabolite is supposed to be the ultimate carcinogen which binds to cellular DNA nonenzymatically to give several quinoline base adducts. Thus Tada and Tada identified four components of the product by paper chromatography; those bound with guanine, termed "QG_I, QG_{II}, and QG_{III}" and the one bound with adenine "QA_{II}" (*3*).

These four adducts can also be prepared by a purely chemical process from a commercial preparation of DNA. Thus the treatment of calf thymus DNA with monoacetyl-4HAQO (1-hydroxy-4-acetoxyimino-1,4-dihydroquinoline), which is readily formed from diacetyl-4HAQO, results in DNA base modification just like that produced enzymatically and in identical adducts at the same ratio in the yield of each adduct (Tada M, Tada M, Huang G-F, et al: Unpublished data). Therefore, one can reasonably deduce that the ultimate metabolite, aminoacyl conjugate of 4HAQO, is formed as 1-hydroxy-4-aminoacyloxyimino-1,4-dihydroquinoline, as shown in text-figure 1 (Tada M, Kawazoe Y: Unpublished data).

The findings we obtained thus far on the structure of these adducts are as follows:

1) QG_I and QG_{II} are barely stable enough to be separated by paper chromatography but are gradually decomposed during the separation procedure while 4AQO is released; these components are difficult to isolate.

2) QA_{II} is stable and easily isolated when the reaction is started with polyA; its structure was determined as 3-(N^6-adenyl)-4-aminoquinoline 1-oxide (*4*).

3) Regardless of enzymatic or nonenzymatic modification of DNA, the main product is 4AQO, overwhelmingly predominant over any other products including DNA quinoline-base adducts, even without any reducing agent present in the medium.

4) QG_{III} is produced only from DNA (not from RNA or homopolymers; Tada M: Unpublished data).

If the chemical reactivity of the ultimate structure, the 4-acyoxyimino derivative of 4HAQO, is taken into account, the reaction scheme illustrated in text-figure 2 can be tentatively assumed as correct; then the experimental results will correlate. The structure of the adducts might be formulated like those shown in text-figure 3. One may further speculate that: 1) Course B predominates in the presence of large amounts of strong nucleophiles; 2) course C predominates; and 3) course A is always minor. As a matter of fact, when monoacetyl-4HAQO is treated with potent nucleophiles such as amines and alkoxide ions, the main products are 3-substituted 4AQO (Kawazoe Y, Araki M: Unpublished data).

Now let us consider which type of base modification is critically responsible for induction of genetic damage. One could deduce that the QA_{II} type of modification is not responsible for such damage, and evidence for such a conclusion includes: 2-Substituted 4NQO's have mutagenicity as potent as 4NQO (Kawazoe Y, Suzuki T, Takahashi K: Unpublished data), although a bulky substituent at the 2-position must substantially decrease the yield of the QA_{II} type of adducts. In addition to this, 3-methyl-4NQO, which has not been demonstrated as being carcinogenic (*5*), is weaker but definitely mutagenic. Potent carcinogenicity of 3-chloro and 3-fluoro 4NQO's might support the idea that substitution in the 3-position of the carcinogen with DNA bases is not responsible for the carcinogenicity. Taking these results into account, one realizes that a bulky 3-substituent in a 4NQO or 4HAQO molecule

Abbreviations: 4HAQO = 4-hydroxyaminoquinoline 1-oxide; 4NQO = 4-nitroquinoline 1-oxide; 4AQO = 4-aminoquinoline 1-oxide.

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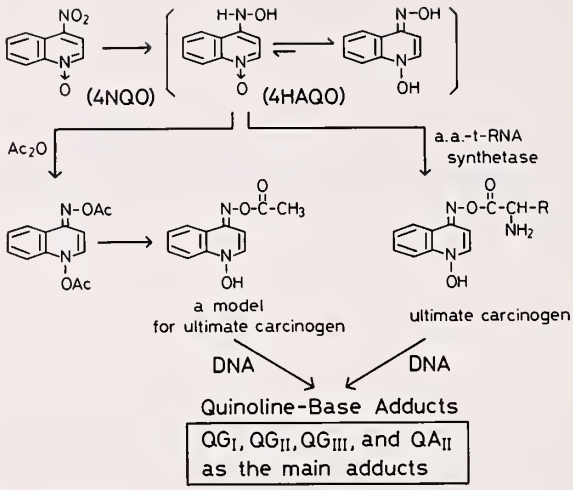
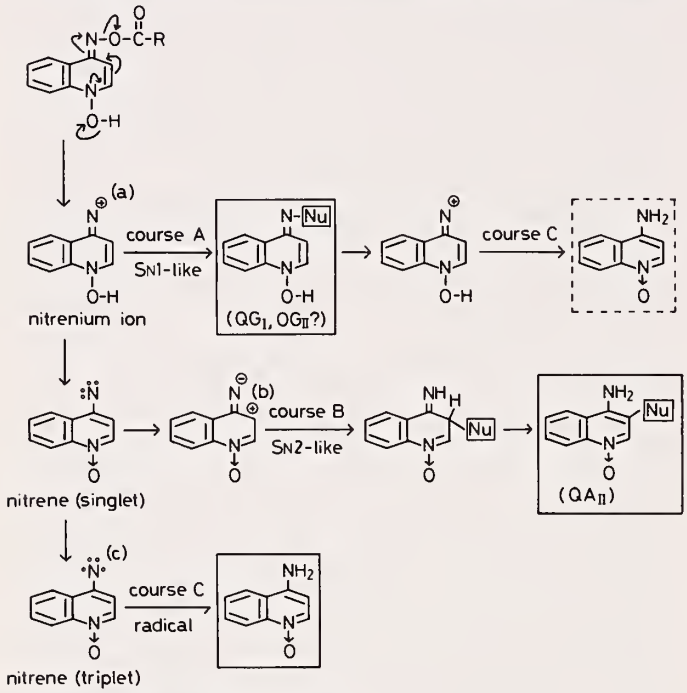
[3] This paper presents the findings of the author's research in cooperation with Dr. Mariko Tada and the late Dr. Mitsuhiko Tada of the Aichi Cancer Center Research Institute, Nagoya; Dr. Misako Araki of the National Cancer Center Research Institute, Tokyo; Dr. Guang-Fu Huang of the Tokyo Biochemical Research Institute, Tokyo; Mr. Kazuhiko Takahashi of Nagoya City University; and some of the author's students at Nagoya City University.



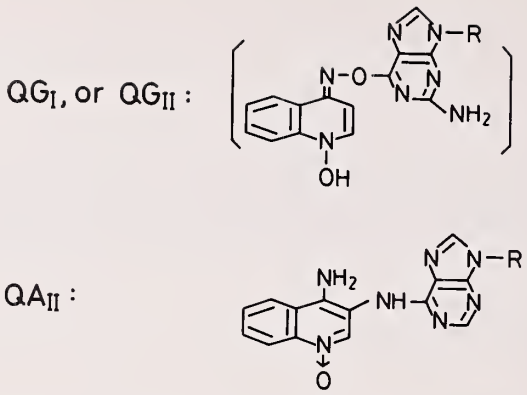
TEXT-FIGURE 1.—Proposed scheme of metabolic activation of 4-HAQO; a.a.-t-RNA synthetase = aminoacyl-tRNA synthetase.



TEXT-FIGURE 2.—Proposed reaction process of 4-acyloxyamino-quinoline 1-oxide.

just decreases quantitatively, but not qualitatively, the carcinogenicity and mutagenicity of the substituent due to its steric hindrance 1) to metabolic activation of nitro to hydroxyamino and/or hydroxyamino to acyoxyamino, and/or 2) to binding of the nitrenium ion (or nitrene) to DNA bases.

Some attention should also be paid to QG adducts; their biologic significance, in addition to their chemical structure, is not known in detail. What we know so far is that these adducts are repaired through the excision repair mechanism in microorganisms and mammalian cells,



TEXT-FIGURE 3.—Proposed structures of some quinoline-DNA base adducts.

which Kondo and associates (*6, 7*) have studied extensively.

One can recognize the difference in chemical nature between the nitrenium intermediate in course A and the carbonium intermediate in course B. Chemically, the carbonium intermediate might be attacked by potent nucleophiles in a bimolecular nucleophilic substitution (S_N2) process and, on the basis of the principle of "hard and soft" acids and bases (*8*), the nitrenium intermediate, which is regarded as a hard electrophile, might be attacked by hard nucleophiles such as oxygen function in a guanine base. It is most important to understand the chemical background of the entire process of DNA modification; i.e., which site of which base is modified. A causal relationship should exist between the quality of the gene damage and the chemical nature of the carcinogen.

REFERENCES

(*1*) TADA M, TADA M: Metabolic activation of 4-nitroquinoline 1-oxide and its binding to nucleic acid. *In* Fundamentals in Cancer Prevention (Magee PN, Takayama S, Sugimura T, et al, eds). Tokyo: Univ Tokyo Press, 1976, pp 217–227

(*2*) ———: Main binding sites of the carcinogen, 4-nitroquinoline 1-oxide, in nucleic acids. Biochim Biophys Acta 454:558–566, 1976

(*3*) NAGAO M, SUGIMURA T: Molecular biology of the carcinogen, 4-nitroquinoline 1-oxide. Adv Cancer Res 23: 131–169, 1976

(*4*) KAWAZOE Y, ARAKI M, HUANG G-F, et al: Chemical structure of QA_{II}, one of the covalently bound adducts of carcinogenic 4-nitroquinoline 1-oxide with nucleic acid bases of cellular nucleic acids. Chem Pharm Bull (Tokyo) 23: 3041–3043, 1975

(*5*) KAWAZOE Y, ARAKI M, NAKAHARA W: The structure-carcinogenicity relationship among derivatives of 4-nitro- and 4-hydroxyamino-quinoline 1-oxide. Chem Pharm Bull (Tokyo) 17:544–549, 1969

(*6*) IKENAGA M, TAKEBE H, ISHII Y: Excision repair of DNA base damage in human cells treated with the chemical carcinogen, 4-nitroquinoline 1-oxide. Mutat Res 43:414–427, 1977

(*7*) IKANAGA M, KAKUNAGA T: Excision of 4-nitroquinoline 1-oxide damage and transformation in mouse cells. Cancer Res 37:3672–3678, 1977

(*8*) Ho T-L: Hard and Soft Acids and Bases Principle in Organic Chemistry. New York: Academic Press, 1977

Discussion IV [1]

E. C. Miller: The session is now open for comments or questions. Dr. Scribner?

J. Scribner: For about the last 3 or 4 years, we have been using S_1 endonuclease and acid phosphatase as degrading enzymes rather than an analytical enzyme. We have consistently obtained clean hydrolysates with essentially complete removal of higher molecular weight material, i.e., clean peaks. The only concession we have to make is that we do have to make the DNA single stranded by heating it a few minutes in boiling water.

So far, we have no reason to believe that that causes us any problems. I think Dr. Weinstein's group has done this too. It might be a good idea generally just to shift away from the alkaline phosphatase and clean phosphodiesterase to an acid system. It may make life a lot easier for all of us.

Kriek: I agree. As I showed in my talk, in the earlier procedure used by many people with the DNA diphosphodiesteration, alkaline phosphatase is not suitable if you want to determine the deoxyguanosine arylamine adduct by whatever procedure, either chromatographically or immunologically. You have to use an acid hydrolysis, either enzymatically or chemically. I might add, we have been using the same method you mentioned. The disadvantage of this procedure is that you cannot determine 2-FAA adducts because deoxyguanosine 2-FAA is, according to our experience, deacetylated completely when S_1 endonuclease and acid phosphatase at pH 5 are used.

E. C. Miller: I would like to call for discussion on Dr. Kadlubar's paper. Yes, Dr. Neumann.

H-G. Neumann: I noticed that we have two different interpretations of what is meant by a bound metabolite fitting well into the small groove. I noticed that Dr. Kadlubar said that this does not cause any perturbation. I understood he meant it as practically a noncritical adduct. In the next paper by Dr. Beland, we had the persistent bound product which also fitted nicely into the small groove. Could we come to some agreement of what the importance is of this kind of interaction?

E. C. Miller: It may be easier to come to agreement on the data than on the importance, I think. Dr. Kadlubar?

F. F. Kadlubar: Well, I do not know if that can be answered. I was not trying to indicate that those exocyclic amino group substitutions were noncritical lesions. All I was indicating is that in our molecular models and in some limited physical chemical studies, they did not appear to cause conformational changes in DNA, and, as such, may fail to be recognized by repair enzymes. Whether they are involved in carcinogenesis by induction of a heritable lesion would depend, I believe, on their ability either to mispair or to misrepair. Maybe they can do that, and maybe that is something we ought to be investigating.

D. Grunberger: I only want to add that my paper deals with this problem, and Dr. Daune and I will discuss this.

E. C. Miller: You mean you are going to solve all the problems for us?

Kadlubar: I want to reply to what Dr. Scribner said. We tried hydrolysis procedures of DNA under neutral conditions, like you suggested, with endonuclease P_1. I am afraid to say that we still get a ring-opened derivative out of that.

More importantly, if we use endonuclease P_1 and acid phosphatase, neither of the amines substituted, i.e., the N-2 or the N-6 adenine adducts, will be released. So endonuclease P_1 seems only to take out the C-8 guanosine, which is another reason we should continue to use the standard alkaline phosphatase.

E. C. Miller: Are there any further comments on Dr. Kadlubar's paper? If not, we will move on to the comments on Dr. Neumann's presentation.

D. Clayson: I am delighted to hear somebody trying to put the whole of this chemistry in the perspective of what is happening biologically. However, I was reminded several times during Dr. Neumann's talk that dimethylaminostilbene, if I remember correctly, has to be given over a considerable period before tumors develop. When you studied proliferation, Dr. Neumann, did you look in the resting untreated tissue, in the tissue after a single administration of the carcinogen, or in something which has continued to be exposed? I think it is possible that if you expose the animal to the carcinogen long enough, you may find something different.

Neumann: My remarks about proliferation are entirely restricted to the effect of a single toxic dose on the glandular stomach. I am inferring only that we find good correlation with the proliferation rate of the different cell populations in the glandular stomach and their susceptibility for acute toxic effects.

G. Williams: Dr. Neumann, what was the timing between the zymbalectomy and the administration of dimethylstilbene?

Neumann: The zymbalectomy was done 2–3 weeks before we added the compound. Because you can only do 2 animals at a time, we did 10 animals in 2.5 weeks, then a rest period followed until they appeared happy, and then we gave them the dimethylaminostilbene.

Williams: I was just wondering if an interesting experiment might be to give the carcinogen first, wait an interval, and then do the zymbalectomy. You postulated that the reason that you got the tumors in the eyelid and lip was due to the fact that initiated cells were there that somehow were able to go on to tumor development in the absence of the

Abbreviation: *N*-2-FAA = *N*-2-fluorenylacetamide.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

development of tumors in the Zymbal's glands. What I am wondering is whether the zymbalectomy did not somehow trigger a proliferative response in these other sebaceous glands that were then a replicative mode when the carcinogen was administered.

Neumann: Your idea would be a good control experiment, but I cannot imagine that there is a proliferation stimulus in this tissue.

E. C. Miller: In that regard, Dr. Neumann, you did not give your dimethylaminostilbene for a period of weeks after your first dose? I think it would tend to dilute an effect of such a study that Dr. Williams suggested. When we studied acetylaminostilbene as a carcinogen, and aminostilbene as a carcinogen, I was impressed that if you compared the doses that you have to use to keep the animals alive, which are much lower than those you use for the fluorene derivatives, the dose for liver tumors was not all that different. Our data were not good, but my impression was that, by studying those doses and given the same doses of aminofluorene or acetylaminofluorene and the same period of survival of the animals, the incidence of liver tumors would not have been high in the fluorene. It is not as much a difference in the sensitivity of the liver as it is a great degree of sensitivity of the Zymbal's glands. We would need better data than we had at that time to make a firm statement.

If there is no further discussion on Dr. Neumann's paper, we can proceed to Dr. Scribner's talk. Dr. Hinson, do you have a question?

J. Hinson: I did not mean to leave you with the impression earlier that ascorbic acid was the only compound that, if used, would increase the mutagenicity, under the conditions of 3′-phosphoadenosine-5′-phosphosulfate, the bacteria, and the supernatant. What we have here are increasing concentrations of our agent. Here is glutathione, this is the ascorbic acid. In the experiments I described, we were using 2 mM.

Also, we have the effect of NADPH and NADH. We also found a reduction of the reactive metabolite back to FAA, approximately the same amount that we found for reduction of ascorbic acid. The short curve indicates that this is almost like an enzymatic effect, rather than a nonenzymatic effect, whereas this may be construed as a nonenzymatic type of effect.

This is further proof that we have a reduction of the reactive metabolite back to FAA. What we presumed would occur here was possibly an enzymatic type of reduction.

E. C. Miller: Is there any further discussion on Dr. Scribner's paper?

J. Weisburger: Dr. Scribner, you may recall that on the basis of the work of Miller, Miller, and Enomoto, as well as Dr. Fiala's work with the smaller arylamines, we postulated that maybe ring epoxidation of 2-FAA may be a factor for subcutaneous carcinogenesis, as promoted by croton oil. Would you care to tell us, on the basis of your calculations, what sort of epoxide we ought to be looking for in FAA?

Scribner: I cannot give you that answer now, I will have to recheck our data. I have to resolve what type of intermediates we are looking for in epoxide formation. I think Lester Shipman, from Oregon, has postulated that, at least with benz[*a*]pyrene, there is indeed an attack of electrophilic oxygen on a double bond. One might also ask whether oxygen attaches to a single atom first, and then an epoxide is formed. These two ideas lead to two conceivably different mechanisms and two calculations.

J. Weisburger: Personally, I speculate it would be on the ring where the amino group is, so that we would get a 3,4-epoxide. On the other hand, Dr. Elizabeth Weisburger visualized the formation of other epoxides.

Scribner: This is the type of thing that one could do. Actually, I believe we do not have a calculation for epoxidation. It is readily approached, and I think we have some ideas going to develop this in the next few months.

R. Floyd: I could not exactly discern the conclusion you drew with reference to the *N*-acetoxy-2-FAA. I can tell you about some experiments we have done that may either add to or clear up the issue as to whether there is a nitrogen radical in the hydrolysis of the *N*-acetoxy-2-FAA.

We have done spin trapping experiments with this compound in a methanol-water combination. We have never found a nitrogen radical. We have never found it where it would normally be clearly shown in the spectra. We have something else, but we do not know exactly what we have. It is not a nitrogen atom.

Scribner: The conclusion I have is that I think this is at least one idea I will have to abandon.

E. C. Miller: I now ask for comments or discussion with regard to Dr. Kawazoe's paper on nitroquinoline *N*-oxide.

Grunberger: I would like to ask Dr. Kawazoe about the activation when one uses aminoacyl-tRNA synthetase. There are many synthetases in most cells, all working with a high fidelity. Which one did you use and how pure was it? Are you using ATP for activation, which is a required condition for synthetases?

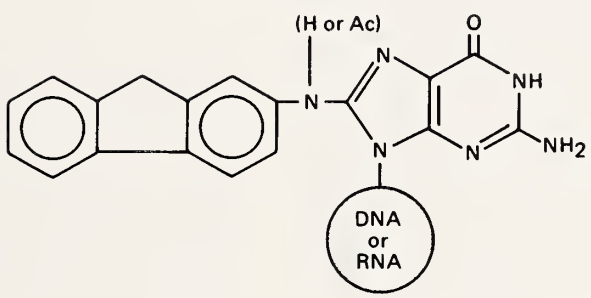
Y. Kawazoe: This enzyme activation study was done by the Drs. Tada. They used yeast enzymes at first, but now they are using liver enzymes. With the yeast enzymes, the serine, followed by choline, aminoacyl-tRNA synthetase seemed the most effective. Mammalian enzymes are different. These studies are being done now.

E. C. Miller: The question was whether you were using ATP.

Kawazoe: Yes, we are.

E. C. Miller: I found this to be a most stimulating session. I thank our speakers and discussants for their interesting contributions.

Session V: Animal Studies: Repair



Session Chairman: Lawrence Grossman

Discussion Chairman: J. Justin McCormick

Enzymatic Mechanisms of DNA Repair[1]

Lawrence Grossman[2]

ABSTRACT—DNA repair proficiency in cells is expressed by various enzymes which can recognize damaged sites arising from exogenous agents or endogenous conditions. Either a damaged base is recognized by DNA glycosylases, partially removed by hemi-DNA glycosylases acting on diadduct damage, or direct incision of the phosphodiester bond near the damaged site. Incision at those apurinic or apyrimidinic sites arising from depurination–depyrimidination or glycosylase reactions is effected by apurinic or apyrimidinic endonucleases. Excision of damaged sites is catalyzed by unique exonucleases followed by DNA polymerase catalyzed reinsertion of nucleotides. The integrity of the strands is restored by polynucleotide ligase when a juxtaposed nucleotide is properly reinserted.—Natl Cancer Inst Monogr 58: 189–192, 1981.

With the variety of chemicals introduced into the environment within the last few decades we can anticipate that cellular resistance to their effects must be accommodated by general classes of DNA repair enzymes. Those bacterial mutants deficient in repair enzymes in addition to exhibiting increased lethality to the damaging agents invariably are more easily mutagenized by the same agents. The "tester" *Salmonella typhimurium* strains used in the rapid bacterial screening procedures are repair-deficient mutants that show a predisposition to mutations by a large variety of carcinogens and mutagens (*1*).

A similar predisposition to carcinogenesis by UV light is seen in DNA repair-deficient patients with the photosensitive disease XP. Primary cell lines derived from these patients are UV sensitive, and they exhibit increased UV-induced mutation rates when compared with presumed heterozygotes for this disease (*2*). Many of the XP cell lines (at least 7 complementation groups exist) show an increased sensitivity to *N*-fluorenylacetamide, benzo[*a*]-pyrene and 7,12-dimethylbenz[*a*]anthracene (*3*). The analogies between repair-deficient XP cell lines, the S. *typhimurium* tester strains and *uvrA, uvrB,* and *uvrC* mutants of *Escherichia coli* are strikingly similar.

Although examples of inducible repair enzymes to alkylation damage have been identified (*4*), the constitutive levels of repair enzymes probably accommodate to newer generations of chemicals the primary effects of which probably result in generic kinds of damage to genetic material. Specificity of DNA repair enzymes can be expected to have evolved in coordination with the introduction of environmental pressures by those physical and chemical agents which specifically damage the genetic apparatus.

A constant source of vulnerability to DNA is its endogeneous environment which, even at physiologic pH's and temperatures, can lead to significant depurination, depyrimidination, and deamination (*5, 6*). The extent of such modifications when extrapolated from conditions in vitro to the cellular content and lifetimes of DNA between replication events must result, if unchecked, in significant structural and informational perturbations. The extent of DNA depurination can be estimated to result in thousands of AP sites per mammalian genome (*7*). Were this much potential DNA damage to remain unchecked, accumulation of noninstructional lesions would eventually be expressed in an aberrant transcriptional flow or perhaps during replication would lead to persistent misinformation. Therefore, a steady state must exist between both repair and those replicational processes that require fidelity of informational content. Although the major surveillance system involving removal and repair can function independently of replication and transcription, these latter systems may overwhelm repair so that post-replicational processess could lead to secondary splicing or recombinational processes expressed in sister chromatid exchanges. The ensuing description will center on excision mechanisms in which damaged bases and nucleotides are selectively removed from perturbed DNA structures.

Text-figure 1 summarizes current perceptions of the roles of specific enzymes in the recognition of damaged nucleotides. At the outset, we should emphasize that the role of damage-specific endonucleases may be restricted to the recognition of AP or APY sites. Such endonucleases assume a strategic locus in repair of spontaneously generated AP and APY sites which arise through depurination or depyrimination or those same sites that arise from the action of a generic group of enzymes referred to as DNA glycosylases (*4–6*). These enzymes assume, perhaps, the most critical position in the initiation of DNA repair processes. Chronologically, the first repair enzyme to be described which acts in such a manner is a DNA glycosylase that recognizes uracil (*8*). This enzyme catalyzes hydrolysis of the N-glycosylic bond linking the N7′ uracil and the C-1′ position of the deoxyribose moiety which liberates free uracil and thereby generates an APY site. This glycosylase can recognize uracil whether or not it is in a hydrogen bond with complementary strands. Uracil which can

Abbreviations: XP = xeroderma pigmentosum; AP = apurinic; APY = apyrimidinic.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

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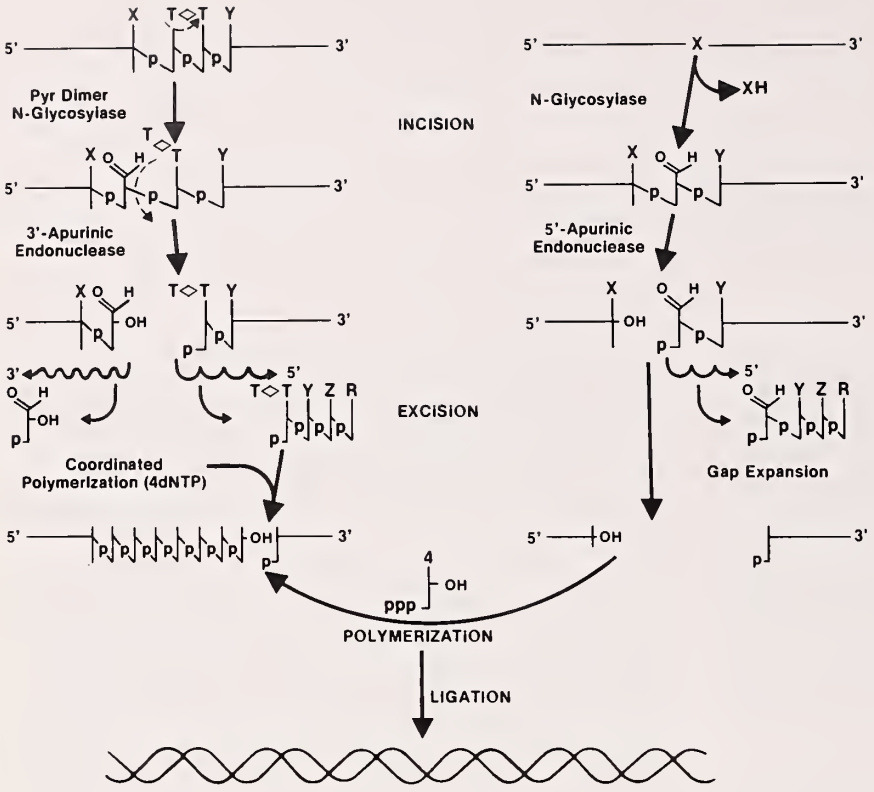

TEXT-FIGURE 1.—Enzymatic pathways of DNA repair. *Left:* nucleotide removal; *right:* base removal.

be misincorporated into DNA in dUTPase mutants (*9*) or which arises through spontaneous deamination of cytosine is monitored by this specific uracil DNA glycosylase.

Uracil DNA glycosylase, like many of the enzymes of this type, will not act on other modified bases such as hypoxanthine, a deamination product of adenine, or 3-methyladenine, all of which are removed by separate and unique DNA gylcosylases. It can be anticipated that a number of enzymes which may act on various dissimilarly modified bases will be detected. When the role of repair of arylated bases can be better understood, they too will probably be repaired by initial DNA glycosylation reactions.

For those bases involved in intrastrand dimerization, such as the pyrimidine cyclobutane dimers arising from UV irradiation, there is a hemi-DNA glycosylase mechanism in which only the 5′ thymine moiety of the thymine-thymine dimer is released from its N-glycosylic bond. The mixed pyrimidine-pyrimidine nucleotide dimer is eventually excised. The net repair reaction is a mixed DNA glycosylase-incision reaction for the release of the damaged nucleotide pair (*10*). From genetic experiments in *E. coli,* Cole and Sinden (*11*) suggested that such a mixed reaction may also be involved in interstrand dimer repair of psoralen-induced cross-linking of DNA.

When APY sites adjacent to a thymine-thymidylate dimer are revealed, the next step in repair involves phosphodiester bond hydrolysis catalyzed by a number of endonucleases specific for these sites. A number of these enzymes appear to have specificities that are not limited to the presence of either a purine or pyrimidine on the strand complementary to the AP sites but rather to the side to which the phosphodiester bond hydrolysis occurs. *E. coli* exonuclease III possesses the first described AP endonuclease activity associated with this enzyme in addition to a 3′ phosphomonoesterase (*12*). These activities are associated with a single enzyme whose gene has been located and identified as *xth* (*13*) in *E. coli.* Since this discovery, other AP endonucleases have been located in *E. coli* (*14*), *Micrococcus luteus* (*15*), calf thymus (*16*), and human placenta (*17*). Although these enzymes are required to initiate repair to endogeneously depurinated DNA, they must also function after DNA glycosylase reactions. Depending on which side of the phosphodiester bond hydrolysis occurs, the nature of the excision reaction will be reflected to a certain degree in the extent to which nucleotides are reinserted. The gap or patch sizes which reflect the nature of DNA damage and, hence, the specific glycosylases acting at such sites, may determine the specific preference for the AP endonuclease that catalyzes phosphodiester hydrolysis.

Excision reactions are catalyzed either by bidirectional exonucleases or those that show a terminal preference. For example, a 3′ AP endonuclease acting after a pyrimidine dimer DNA glycosylase reaction results in a nick with damaged nucleotides at both the 3′ and 5′ termini. Under these circumstances, those nucleotides 3′ and 5′ to the nick require excision prior to reinsertion reactions by DNA polymerases. In both bacteria and mammalian cells, bidirectional exonucleases show catalytic potential in removal of these sites by virtue of their preference for single-stranded regions, their ability to act at nicked duplexes bearing noncomplementary termini, or by virtue of their ability to act at internal and terminal nucleotides. Exo-

nuclease VII of *E. coli* (*18*), the UV exonuclease of *M. luteus* (*19*), and a similar type of enzyme isolated from human placenta (*20*) possess these inherent excision properties. Bacterial DNA polymerase type I enzymes in addition to their 5′ → 3′ polymerizing capabilities possess two exonucleolytic functions that can participate in repair processes. Existing on the same polypeptide is a 3′ → 5′ exonuclease which prefers single-stranded DNA, acts on terminal nucleotides, and is involved in "editing" noncomplementary nucleotides at the growing end of the nascent DNA chain (*21*). The enzyme also possesses a 5′ → 3′ exonuclease which can initiate hydrolysis on the duplex structure by acting at internal phosphodiester bonds and removing damaged nucleotides in the same direction simultaneously with reinsertion of the newly reinserted nucleotides. This efficient coordinated process is not duplicated in eukaryotes whose DNA polymerases are devoid of associated exonucleotic activities, and hence it depends on independent exonucleases to function in repair.

Although a bidirectional exonuclease has been identified in human tissues, other exonucleases can fulfill excision needs in either the 3′ (Hollis G, Grossman L: Manuscript in preparation) or 5′ direction (Pedrini AM, Grossman L: Manuscript in preparation).

The reinsertion reactions are ideally suited for DNA polymerase I, not only because it can coordinate excision and reinsertion but because it has a greater affinity for nicked DNA than the other DNA polymerases in bacteria. Similarly in mammalian cells, the DNA polymerase β can bind to nicks or small gaps (Korn D: Personal communication). The DNA polymerase α, implicated in replication, can also fulfill reinsertion reactions at a single-strand gap of 20–70 nucleotides (*22*) and may participate in repair.

The final stages of repair require restoration of the integrity of the damaged strand. This restoration is accomplished by the enzyme polynucleotide ligase which, in the presence of NAD^+ in bacteria or ATP in eukaryotes and phage-infected bacteria, can form the final phosphodiester bond between the 5′ phosphorylated terminus and a juxtaposed 3′ hydroxyl nucleotide (*23*). This enzyme fortuitously can also restrict intensive nick translation in bacteria because of the coordinated 5′ excision in 5′ → 3′ directed polymerization and assume a similar role in mammalian cells.

This overall review is not designed to be exhaustive but to give some perceptions of the current enzymologic capabilities of cells in their recognizing damaged nucleotides and restoring the rightful sequence of nucleotides and integrity to the strands. What has not been discussed is the mechanism of postreplication repair in which gaps are formed opposite noninstructional lesions (*24*). Polymerization proceeds beyond the point of the damaged site that generates nascent strands with breaks which, through recombination mechanisms yet to be characterized enzymatically, result in exchange of strands. It is also apparent from work in vivo that many damaged nucleotides persist in daughter cells and remain during the replication process.

That many of the carcinogens described in detail during these meetings have as their target those cells which rapidly proliferate is more than coincidental. Perhaps the repair process is considerably slower than that of replication, and, during the replication phase, damage may escape the repair process and be integrated into functioning DNA strands that eventually lead to aberrations that influence informational flow.

REFERENCES

(*1*) Ames BW: Identifying environmental chemicals causing mutations and cancer. Science 204:587–593, 1979

(*2*) Kraemer K: Progressive degenerative diseases associated with effective DNA repair and xeroderma pigmentosum and ataxia telangiectasia. *In* DNA Repair Processes (Nichols WW, Murphy DG, eds). Miami: Symposia Specialists, 1977, pp 37–71

(*3*) Amacher DE, Lieberman MW: Removal of acetylaminofluorene from the DNA of control and repair-deficient human fibroblasts. Biochem Biophys Res Commun 74: 285–290, 1977

(*4*) Samson L, Cairns J: A new pathway for DNA repair in *Escherichia coli.* Nature 267:281–283, 1977

(*5*) Lindahl T, Nyberg B: Rate of depurination of native deoxyribonucleic acid. Biochemistry 11:3610–3618, 1972

(*6*) Lindahl T, Karlstrom O: Heat-induced depyrimidation of deoxyribonucleic acid in neutral solution. Biochemistry 12:5151–5154, 1973

(*7*) Lindahl T: DNA repair enzymes acting on spontaneous lesions in DNA. *In* DNA Repair Processes (Nichols WW, Murphy DG, eds). Miami: Symposia Specialists, 1977, pp 225–240

(*8*) _____: An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proc Natl Acad Sci USA 71: 3649–3653, 1974

(*9*) Tye BK, Nyman PO, Lehman IR, et al: Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. Proc Natl Acad Sci USA 74: 154–157, 1977

(*10*) Haseltine WA, Gordon LK, Lindan CP, et al: Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of *M. luteus.* Nature 285:634–641, 1980

(*11*) Cole RS, Sinden RR: On the mechanism for repair of cross-linked DNA in *E. coli* treated with psoralen and light. *In* DNA Repair Mechanisms (Hanawalt PC, Friedberg EC, Fox EF, eds). New York: Academic Press, 1978, pp 287–290

(*12*) Weiss B: Endonuclease II of *Escherichia coli* is exonuclease III. J Biol Chem 251:1896–1901, 1976

(*13*) Yajko DM, Weiss B: Mutations simultaneously affecting endonuclease II and exonuclease III in *Escherichia coli.* Proc Natl Acad Sci USA 72:688–692, 1975

(*14*) Linquist S: A new endonuclease from *Escherichia coli* acting at apurinic sites in DNA. J Biol Chem 252: 2808–2814, 1977

(*15*) Laval J: Two enzymes are required for strand incision in repair of alkylated DNA. Nature 269:829–832, 1977

(*16*) Lindahl T: DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog Nucleic Acid Res Mol Biol 22:185–192, 1979

(*17*) Shaper NL, Grossman L: Purification and properties of the human placental apurinic/apyrimidinic endonuclease. *In* Methods in Enzymology (Grossman L, Moldave K, eds). New York: Academic Press, 1980, pp 216–224

(*18*) Chase JW, Richardson SS: Exonuclease VII of *Escherichia coli.* J Biol Chem 249:4553–4561, 1974

(*19*) Kaplan JC, Kushner SR, Grossman L: Enzymatic repair of DNA III. Biochemistry 10:3315–3324, 1971

(*20*) Doniger J, Grossman L: Human correxonuclease: Purification and properties of a DNA repair exonuclease from placenta. J Biol Chem 251:4579–4587, 1976

(*21*) Muzyczka N, Poland RC, Bessman JR: Studies on the biochemical basis of spontaneous mutation. J Biol Chem 247:7116–7122, 1972

(22) Korn D, Fisher PA, Battery J, et al: Structural and enzymological properties of human DNA polymerases alpha and beta. Cold Spring Harbor Symp Quant Biol 43:613–624, 1978

(*23*) Konrad EB, Modrich P, Lehman IR: Genetic and enzymatic characterization of a conditional lethal mutant of *Escherichia coli* K12 with a temperature-sensitive DNA ligase. J Mol Biol 75:519–529, 1973

(*24*) Witkin EM: Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli.* Bacteriol Rev 40:869–907, 1976

N-2-Fluorenylacetamide-Induced Conformational and Functional Damage to DNA[1, 2]

Dezider Grunberger[3, 4]

ABSTRACT—Conformational and functional changes induced in DNA by *N*-2-fluorenylacetamide (2-FAA) modification are described. The carcinogen was bound to the C-8 and to N^2 positions of guanosine (G) residues and also formed a deacetylated *N*-2-fluorenamine (2-FA) adduct. Modification of G residues at the C-8 position caused a large conformational distortion in DNA expressed in a "base displacement model." According to this model, the G residue rotated from the preferred *anti* to *syn* positions, and the bulky 2-FAA residue was inserted into the DNA helix and displaced the G residue. The conformational distortion in DNA was detectable by single-strand specific S_1 nuclease digestion. On the other hand, substitution of 2-FAA on the N^2 position of G did not produce a major change in conformation of the DNA helix, and this was not recognized by S_1 nuclease splitting. On the basis of circular dichroism, proton magnetic resonance, and S_1 nuclease digestion data, it seemed that 2-FA-induced damage to DNA was less than that induced by 2-FAA modification of G at the C-8 position. Finally, effects induced by 2-FAA on RNA synthesis in vitro and in vivo systems and the possible relationship between the repair mechanisms and conformational damage of DNA are discussed.—Natl Cancer Inst Monogr 58: 193–199, 1981.

There is increasing evidence that covalent binding of carcinogens to cellular macromolecules, particularly nucleic acids, is the initial critical event in the encounter between an environmental carcinogen and target cells in the exposed host (*1, 2*). Therefore, to understand carcinogenesis at a molecular level, one must determine the complete chemical structure of the carcinogen–macromolecular adducts and the associated conformational changes in the target macromolecules. On this basis, one can relate the chemical and physical alterations to aberrations in the functional properties of the chemically modified macromolecules.

A complicating feature in one's attempts to relate chemical structure to functional effects is the fact that with most of these agents more than one type of nucleoside adduct in DNA is formed. This is also true with the simpler alkylating agents in which almost every nitrogen and oxygen residue of all the nucleic acid bases can be modified (*3*). In their work with 2-FAA, Miller et al. (*4*) first demonstrated that both RNA and DNA can react nonenzymatically in vitro with *N*-AcO-2-FAA. The major product obtained from hydrolysates of the modified DNA is *N*-(deoxyguanosin-8-yl)-FAA (*5*), and a minor component is 3-(deoxyguanosin-N^2-yl)-FAA (*6, 7*). These same nucleic acid derivatives were found in rat liver DNA when 2-FAA was administered in vivo (*6*). However, after in vivo application of *N*-OH-2-FAA, about 80% of the major adduct was in the deacetylated form (*8–10*). Some studies suggest that in addition to the guanine adducts, 2-FAA may also modify adenine residues in nucleic acids (*11–13*), although this adduct has not actually been isolated and characterized. Inasmuch as modification of the C-8 or N^2 positions of the G residue in DNA could be associated with different conformational changes, we studied the conformations of both types of adducts.

CONFORMATIONAL DAMAGE TO DNA

Guanosine C-8–*N*-2-Fluorenylacetamide Adduct

Fundamental to an understanding of the functional consequences of nucleic acid modification by the bulky carcinogens such as 2-FAA is information on two points: 1) the orientation within a single- or double-stranded nucleic acid of the covalently bound carcinogen residues, and 2) possible alterations in the native conformation of the nucleic acid that result from this modification. In the modification of the C-8 position of guanine residues in double-stranded DNA by 2-FAA, considerable evidence has been obtained for a conformational distortion we termed "base displacement model" (*14, 15*). According to this model and for accommodation of the bulky 2-FAA residue, modification of the C-8 position of G requires rotation of the base about the glycosidic bond of deoxyguanosine from the preferred *anti* to the *syn* position (*16, 17*).

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; *N*-AcO-2-FAA = *N*-acetoxy-2-FAA; *N*-OH-2-FAA = *N*-hydroxy-2-FAA; G = guanosine; 2-FA = 2-fluorenamine.

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[2] Supported by Public Health Service grants CA21111 and CA13696 from the National Cancer Institute.

[3] Departments of Biochemistry and Environmental Sciences, Columbia University College of Physicians and Surgeons, and the Cancer Center-Institute of Cancer Research, New York, N.Y. 10032. *Address reprint requests to* Dr. Grunberger, Hammer Health Sciences Building 1024, 701 W. 168th Street, New York, N.Y. 10032.

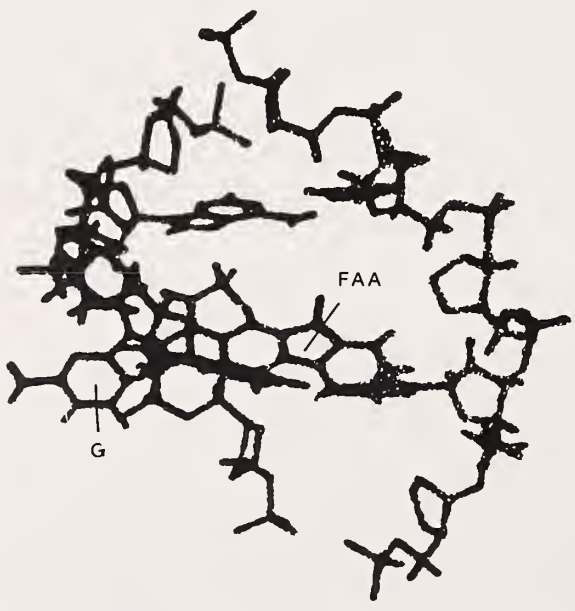
[4] I acknowledge the collaboration of Drs. I. B. Weinstein, P. Pulkrabek, H. Yamasaki, R. Santella, E. Kriek, and F. L. Yu in these studies; of Dr. L. Katz for performing the computer graphics; and of Ms. Sue A. Allen in the preparation of the manuscript.

These changes are best illustrated in a computer-generated display of a double-stranded DNA fragment (text-fig. 1). The computer display allows one to perform rotation around appropriate bond angles readily while a three-dimensional image of the molecular structure is being obtained on a video screen. Text-figure 1 displays the conformation of a double-stranded region containing 3 hydrogen-bonded base pairs with 1 G modified on the C-8 position with a 2-FAA residue. In the display, the guanine base has been rotated around the glycosidic bond to the *syn* conformation to avoid the steric hindrance. In addition, the planar fluorene ring system is inserted into the helix occupying the former position of the displaced guanine residue. The G residue displaced by 2-FAA in the double helix evidently cannot base-pair with the C residue on the complementary strand, and during the process of replication or transcription, no base-pairing at this position could occur. We have obtained additional evidence of the base displacement model from proton magnetic resonance and circular dichroism spectra of modified oligonucleotides (*17, 18*). A similar model, called the "insertion-denaturation model" has been proposed by Fuchs and Daune (*19, 20*).

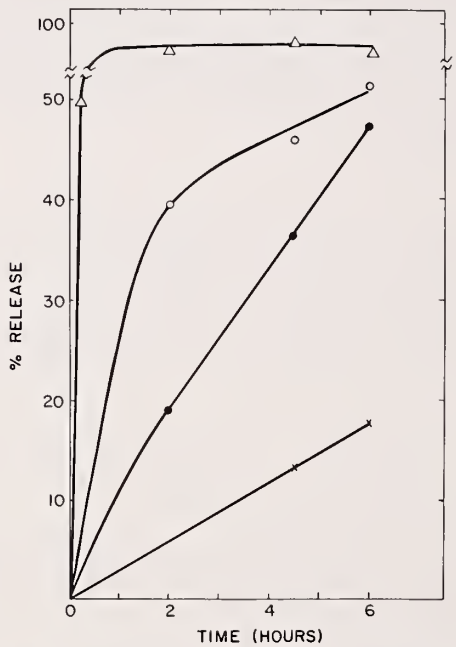
The conformational changes cause a marked distortion of the double-stranded DNA helix at sites of modification by 2-FAA. The best evidence for localized regions of denaturation comes from the studies on the susceptibility of 2-FAA-modified DNA to digestion by S_1 nuclease, a single-strand specific endonuclease from *Aspergillus oryzae* (*21, 22*). When the kinetics of S_1 nuclease digestion of native and heat-denatured duck reticulocyte DNA and of [^{14}C]2-FAA-modified DNA samples are measured, heat-denatured DNA is rapidly and completely hydrolyzed as expected, whereas digestion of native DNA proceeds slowly and to a lesser extent (text-fig. 2). Digestion of [^{14}C]2-FAA-modified DNA kinetic data are intermediate between those of native and heat-denatured DNA. Text-figure 2 also illustrates the release of [^{14}C]2-FAA residues during the splitting of DNA; the release of these residues is faster than that of total nucleotides (A_{260}), which indicates preferential hydrolysis of the [^{14}C]2-FAA regions modified by the labeled carcinogen.

These results indicated that modification of native DNA by covalent attachment of 2-FAA residues led to localized regions of denaturation, inasmuch as the modified regions were excised by S_1 nuclease. The estimated number of base pairs destabilized by a single 2-FAA modification is in the range of 5 to 50, depending on the extent of modification of the DNA, the length of the nuclease digestion period, and the NaCl concentration during the digestion (*23*). Whereas attachment of 2-FAA to G residues requires rotation of the base about the glycosidic bond and there is less hindrance to the rotation of bases in single- than in double-stranded regions, one would predict that G residues in single-strand regions will be more accessible to modification.

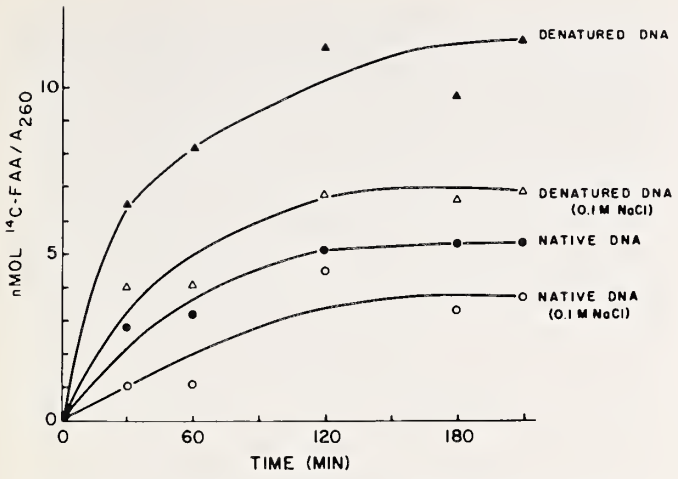
Evidence that single-stranded regions of nucleic acids are more susceptible to modification by 2-FAA than are the double-stranded has been obtained by a comparison of the reactivity of native and denatured calf thymus DNA



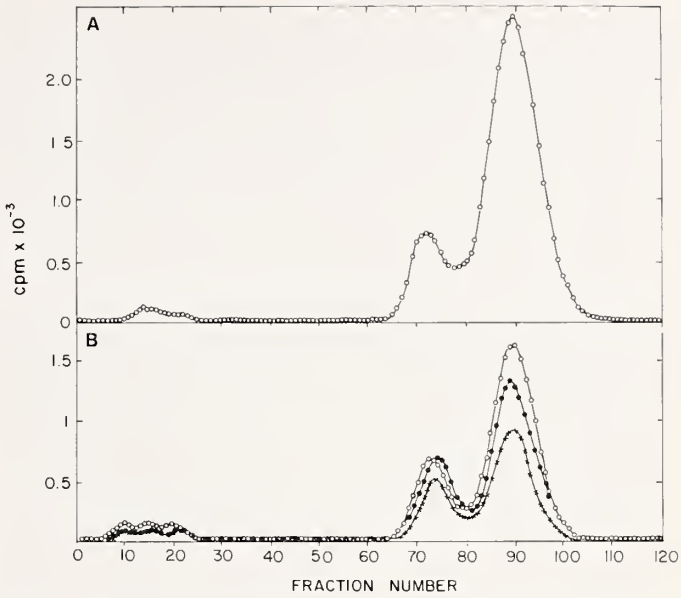
TEXT-FIGURE 1.—Computer-generated three-dimensional structure of a double-stranded DNA fragment with a deoxyguanosine residue modified at the C-8 position by 2-FAA. The G is rotated out from the helix and 2-FAA is inserted in its position.



TEXT-FIGURE 2.—Digestion of [^{14}C]2-FAA-modified DNA with S_1 nuclease. Native, heat-denatured, and [^{14}C]2-FAA-modified DNA were incubated with S_1 nuclease for the indicated periods. *Open triangles* = A_{260} released from heat-denatured DNA; X = A_{260} released from native DNA; *solid circles* = A_{260} released from modified DNA; *open circles* = [^{14}C]2-FAA residues released from modified DNA. For details, *see* (*22*). Text-figure is reproduced with the permission of the publisher (*22*).



TEXT-FIGURE 3.—Effect of NaCl solution on the rate and extent of native and denatured DNA modification by *N*-AcO-2-FAA. Addition of NaCl was 10 min prior to that of the carcinogen. For details, *see* (*24*). Text-figure is reproduced with the permission of the publisher (*11*).



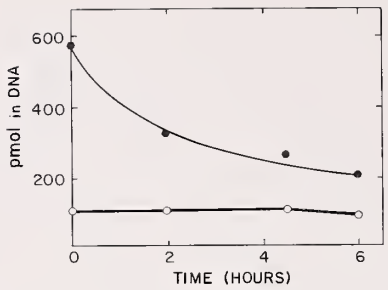
TEXT-FIGURE 4.—A) Sephadex LH-20 column chromatography of a nucleoside hydrolysate of [^{14}C]2-FAA-modified DNA. One A_{260} U of DNA (equivalent to 150 nmol nucleotide residues) containing 700 pmol [^{14}C]2-FAA was completely hydrolyzed by a series of enzymes; then the hydrolysate was chromatographed on a Sephadex LH-20 column, and radioactivity was measured. For details, *see* (*22*). B) Sephadex LH-20 column chromatography of nucleoside hydrolysates obtained from the S_1 nuclease-resistant fraction of [^{14}C]2-FAA-modified DNA. Modified DNA was incubated with S_1 nuclease for different periods. Nondigested fraction of DNA was precipitated, completely hydrolyzed to nucleosides, and the total hydrolysate was chromatographed on Sephadex LH-20 columns as in A. S_1 nuclease digestion: *open circles* = 2 hr; *closed circles* = 4.5 hr; X = 6 hr. For details, *see* (*22*). Text-figure is reproduced with permission of the publisher (*22*).

to *N*-AcO-2-FAA (*11*). Denatured DNA is approximately twice as susceptible to modification by 2-FAA as is native DNA (text-fig. 3). Inasmuch as media of high ionic strength are known to stabilize the secondary structure of nucleic acids, an increase in ionic strength decreases the reactivity of native DNA with *N*-AcO-2-FAA. A similar effect has been observed in the reaction of this derivative with duplexes between synthetic polydeoxynucleotides (*25*). Preferential modification of single-stranded regions in tRNA supports the proposed model (*26, 27*).

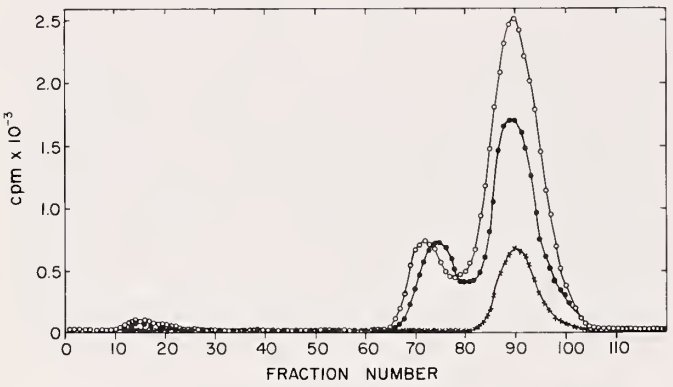
CONFORMATION OF GUANOSINE *N*-2-FLUORENYLACETAMIDE ADDUCT

Because differences have been observed in the steric aspects connected with the modification of C-8 and N^2 positions of G, differences might also exist between the conformational distortions in the DNA helix associated with these two types of adducts. We (*22*) observed that during the course of S_1 nuclease digestion, a change occurred in the ratio of the 2-FAA adducts in the S_1 nuclease-resistant fraction of the DNA (text-fig. 4). When the modified DNA was exposed to S_1 nuclease, the undigested fraction was hydrolyzed, and the modified nucleosides were separated on a Sephadex LH-20 column, it was evident that during a 6-hour incubation with S_1 nuclease the amount of *N*-(deoxyguanosin-8-yl)-FAA in the S_1 nuclease-resistant fraction was progressively and markedly decreased. Only a minute loss of the 3-(deoxyguanosin-N^2-yl)-FAA residues was observed. The Sephadex LH-20 chromatography results, summarized quantitatively in text-figure 5, indicated that S_1 nuclease preferentially digested regions of the DNA containing *N*-(deoxyguanosin-8-yl)-FAA residues, whereas sites with 3-(deoxyguanosin-N^2-yl)-FAA residues remained basically intact. To confirm that the oligonucleotides in single-stranded regions contain mainly *N*-(deoxyguanosin-8-yl)-FAA, we used another single-strand specific endonuclease from *Neurospora crassa*. After incubation, the undigested fraction of the DNA was precipitated with cold ethanol. This material and the released oligonucleotides present in the supernatant fraction were then separately hydrolyzed to nucleosides and analyzed by Sephadex LH-20 column chromatography (text-figure 6). The profile of the undigested fraction of the DNA demonstrated a decrease in the ratio of the C-8 to N^2 adducts of G–FAA. In addition, the C-8 but not the N^2 adduct was detected in the fraction released by the *N. crassa* enzyme. Thus the latter enzyme, like the S_1 nuclease, recognized the regions modified by *N*-(deoxyguanosin-8-yl)-FAA but not those modified by 3-(deoxyguanosin-N^2-yl)-FAA as single-stranded regions on 2-FAA modified DNA molecules.

In contrast to the C-8 adduct, substitution of FAA on the N^2 position of guanine apparently does not produce a major change in conformation of the DNA helix. Although the precise conformation of the helix at the latter sites has not been determined, model-building studies indicated that the N^2 position, in contrast to that of the C-8, of guanine was readily susceptible to chemical modification, and the fluorene residue could simply occupy the minor



TEXT-FIGURE 5.—The number of picomoles of *N*-(deoxyguanosin-8-yl)-FAA (*closed circles*) and (deoxyguanosin-N^2-yl)-FAA (*open circles*) remaining in the S_1 nuclease-resistant fractions obtained from equivalent samples (1 A_{260} unit) of [^{14}C]2-FAA-modified DNA following various incubation times with S_1 nuclease. Values are calculated from the results of the experiment depicted in text-figure 4. For details, *see* (*22*). Text-figure is reproduced with the permission of the publisher (*22*).



TEXT-FIGURE 6.—Sephadex LH-20 column chromatography of nucleoside hydrolysates of the total, the *N. crassa* nuclease-digested fraction, and the *N. crassa* nuclease-resistant fraction of [^{14}C]2-FAA-modified DNA. Hydrolysis to nucleosides and chromatography of the *N. crassa*-resistant and *N. crassa*-digested fractions of DNA were performed as described in (*22*). *Open circles* = total [^{14}]2-FAA-modified DNA; *closed circles* = *N. crassa* nuclease-resistant fraction; X = *N. crassa* nuclease-digested fraction. For details, *see* (*22*). Text-figure is reproduced with the permission of the publisher (*22*).

groove of the DNA helix. Thus the base displacement model may apply only to the C-8 adduct of FAA.

CONFORMATION OF GUANOSINE-2-FLUORENAMINE ADDUCT

Because about 80% of the C-8 of the G adduct is in deacylated form after in vivo application of *N*-OH-2-FAA, one should also investigate conformational changes induced by 2-FA residues. Using the S_1 nuclease splitting technique, Kriek and Spelt (*28*) have recently shown that 2-FA-modified DNA was hydrolyzed three times more slowly than DNA modified by 2-FAA under the same reaction conditions.

Similarly, circular dichroism and proton magnetic resonance spectra of a simple DNA model compound, 2′-deoxyadenylyl-(3′-5′)-2′-deoxyguanosine, modified either by 2-FA or 2-FAA showed less of a stacking interaction between 2-FA and the neighboring A than between 2-FAA and A (*29*). Taken together, these results and the space filling models imply that binding of 2-FA residue to C-8 of G in contrast to 2-FAA does not require rotation of G about the glycosidic bond from *anti* to *syn* conformation and also suggest that the local regions of denaturation caused by 2-FA modification on DNA molecules are smaller than those with 2-FAA modification. Thus damage to DNA induced by 2-FA seems to be less than that induced by 2-FAA on the C-8 and greater than that induced on the N^2 position of G residues.

FUNCTIONAL DAMAGE

The base displacement model predicts that G residues in nucleic acids altered by 2-FAA cannot participate in base-pairing. This was tested in an in vitro translational system with triplet condons and polynucleotides containing 2-FAA-modified G residues as mRNA. A complete inactivation of the coding function of these triplets was found (*16, 30*).

Similar effects were observed during transcription of a 2-FAA-modified DNA template. Troll et al. (*31*) found that mammalian DNA modified with 2-FAA in vitro was impaired in its template activity for bacterial RNA polymerase. The transcription of 2-FAA-modified bacteriophage T7 DNA by RNA polymerase was also markedly inhibited (*32*). Analysis of the RNA products demonstrated that this inhibition is due to premature chain termination, probably because of failure of base-pairing when, during chain elongation, the polymerase encounters modified G residues. The effect of 2-FAA on RNA synthesis in vivo was investigated after administration of *N*-OH-2-FAA to rats (*33, 34*). Two hours after the injections, nuclei were isolated, and nuclear and nucleolar RNA syntheses were measured with the use of endogenous DNA or exogenous poly(dI-dC) as templates (*34*). Table 1 shows that with the endogenous template, RNA synthesis by nuclei from treated animals was inhibited 57%. However, when poly(dI-dC) was used as a template and the endogenous template function was blocked by dactinomycin, the RNA polymerase activity was slightly but consistently increased above that of the untreated control. These results indicated that the inhibition of RNA synthesis was partly due to impairment of the enodogenous DNA template function. Inasmuch as nuclei contain at least three types of RNA polymerases, e.g., nucleolar RNA polymerase I responsible for the synthesis of rRNA, RNA polymerase II of the nucleoplasm in which the mRNA is formed, and RNA polymerase III which catalyzes the synthesis of tRNA, we isolated the nucleolar and nucleoplasmic fractions. When the complete nucleolar fraction containing the enzyme and chromatin was used as the template (table 2), *N*-OH-2-FAA treatment inhibited rRNA synthesis by 80%, but the nucleolar RNA polymerase activity was decreased by only 20% when measured in the presence of dactinomycin with the exogenous poly(dI-dC) as template. These results con-

firmed the previous findings that inhibition of rRNA synthesis is due to the decreased template activity of DNA in rats treated with *N*-OH-2-FAA, which is consistent with the conformational changes generated by the binding of 2-FAA to G residues.

To determine the effect of *N*-OH-2-FAA treatment on mRNA synthesis, we isolated total nuclear RNA polymerase and measured the enzyme activity in the presence and absence of α-amanitin which selectively inhibits RNA polymerase II, the enzyme responsible for mRNA synthesis. Table 3 shows that treatment with *N*-OH-2-FAA resulted in a slight decrease in the enzyme activity. However, we noticed that the RNA polymerase activity from the treated group was almost totally insensitive to α-amanitin. Column "A–B" in table 3 indicates that RNA polymerase II activity alone was inhibited by 66% among the total nuclear RNA polymerase after treatment. The data in column "B" also indicate that the activity of the rest of the RNA polymerase species that were insensitive to a low concentration (3.2 μg/ml) of α-amanitin was in fact slightly increased. Because the RNA polymerase species insensitive to α-amanitin inhibition are RNA polymerases I and III and polymerase I was not increased after *N*-OH-2-FAA treatment (table 2), we may reasonably conclude that the RNA polymerase III species were selectively increased as a result of carcinogen treatment.

A conclusion suggested by these results is that because *N*-OH-2-FAA had little effect on RNA polymerase I activity, the inhibition of nucleolar RNA synthesis is probably due to impairment in function of the nucleolar DNA template. On the other hand, the decrease in nucleoplasmic RNA synthesis may be due to direct inhibition of the enzyme activity of RNA polymerase II, a conclusion supported by the observations of others (*24, 35, 36*).

TABLE 1.—*Effect of* N-*OH-2-FAA injected in vivo on activity of rat liver nuclear RNA polymerase measured in vitro with endogenous and exogenous templates* [a]

Treatment group	RNA polymerase activity, pmol [^{14}C]GMP incorporated/mg DNA			
	Endogenous template	Percent	Poly (dI-dC)	Percent
Control	1,130 ± 81	100	2,539 ± 596	100
N-OH-2-FAA	488 ± 51	43	3,327 ± 476	131

[a] Injections of 3 mg *N*-OH-2-FAA/100 g body wt were given ip 2 hr before rats were killed. Nuclei were isolated by a hypertonic sucrose method and assayed under conditions described in (*34*). Values given are the means of quintuplet determination in each of 3 experiments. Mean ± SE.

TABLE 2.—*Effect of* N-*OH-2-FAA injected in vivo on activity of rat liver nucleolar RNA polymerase measured in vitro with endogenous and exogenous templates* [a]

Treatment group	RNA polymerase activity, pmoles [^{14}C]GMP incorporated/mg DNA			
	Endogenous template	Percent	Poly (dI-dC)	Percent
Control	15,174 ± 174	100	23,723 ± 278	100
N-OH-2-FAA	3,138 ± 385	21	18,238 ± 2,461	77

[a] Experimental conditions were the same as in table 1. Values given are the means of quintuplet determinations in each of 2 experiments. For details, *see* (*34*). Mean ± SE.

TABLE 3.—*Effect of* α-*amanitin on solubilized normal and* N-*OH-FAA-treated rat hepatic nuclear RNA polymerase activity* [a]

Treatment group	RNA polymerase activity, pmol [^{14}C]GMP incorporated/g liver					
	$-\alpha$-Amanitin A	Percent	$+\alpha$-Amanitin B	Percent	α-Amanitin sensitive A − B	Percent
Control	2,125 ± 459	100	1,274 ± 181	100	851	100
N-OH-2-FAA	1,700 ± 223	80	1,408 ± 260	111	292	34

[a] Nuclear RNA polymerase was extracted, and 3.2 μg α-amanitin/ml was added to the reaction mixture at zero time and assayed in the presence of poly(dI-dC) as described in (*34*). Values given are the means of quintuplet determinations in each of 4 experiments. Mean ± SE.

CONCLUSION

With the in vivo significance of our results, Kriek's (*6*) findings that the C-8 adduct is rapidly removed from rat liver in vivo (half-life, 7 days), whereas the guanine-N^2 adduct remained persistently bound to DNA are particularly interesting. However, we have no information on the rate of removal of the G adducts from DNA modified by 2-FA; from the level of DNA damage we could predict that it will be intermediate between the rates of C-8 and N^2 modified G–2-FAA adducts.

Taken together, these in vivo experiments and our in vitro results suggest that the enzyme system of DNA excision repair preferentially recognizes and excises lesions associated with major distortions in conformation of the DNA helix. If one assumes that the DNA excision repair system operates with high fidelity, persistent carcinogen substituents, such as adducts on the N^2 position of guanine, might be more significant in carcinogenesis, i.e., carcinogen potency might be a function of two factors: 1) ability to bind to DNA and alter its template function, and 2) ability to bind in a form that does not produce a conformational distortion readily recognized and excised by error-free DNA repair systems.

It is also possible, however, that carcinogens do not act simply by producing errors in DNA replication at the sites at which they damage DNA. In bacteria, chemical carcinogens induce a highly pleiotropic response called "SOS functions" (*37, 38*). Mutagenesis, one of these functions, appears to result from the induction of an error-prone DNA replication mechanism. It is not yet clear that similar responses to DNA damage occur in eukaryotic cells, although recent experiments support this possibility (*39–41*). Thus further studies are required if the possible role in the carcinogenic process of inducible DNA repair and error-prone DNA synthesis mechanisms is to be determined.

REFERENCES

(1) Miller EC: Some current perspectives on chemical carcinogenesis in humans and experimental animals: Presidential Address. Cancer Res 38:1479–1496, 1978

(2) Grunberger D, Weinstein IB: Biochemical effects of the modification of nucleic acids by certain polycyclic aromatic carcinogens. *In* Progress in Nucleic Acid Research and Molecular Biology (Cohn WE, ed), vol 23. New York: Academic Press, 1979, pp 105–149

(3) Singer B: All oxygens in nucleic acids react with carcinogenic ethylating agents. Nature 264:333–339, 1976

(4) Miller EC, Juhl U, Miller JA: Nucleic acid guanine: Reaction with the carcinogen *N*-acetoxy-2-acetylaminofluorene. Science 153:1125–1127, 1966

(5) Kriek E, Miller JA, Juhl U, et al: 8-(*N*-2-Fluorenylacetamido) guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide in neutral solution. Biochemistry 6:177–182, 1967

(6) Kriek E: Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA in vivo. Cancer Res 32:2042–2048, 1972

(7) Westra JG, Kriek E, Hittenhausen H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149–164, 1976

(8) Irving CC: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26: 1390–1396, 1966

(9) King CM, Phillips B: *N*-Hydroxy-2-fluorenylacetamide. Reaction of the carcinogens with guanosine, ribonucleic acid, and protein following enzymatic deacetylation or esterification. J Biol Chem 244:6209–6216, 1969

(10) Kriek E: Difference in binding of 2-acetylaminofluorene to rat liver deoxyribonucleic acid and ribosomal ribonucleic acid in vivo. Biochim Biophys Acta 161:273–275, 1968

(11) Levine AF, Fink LM, Weinstein IB, et al: Effect of *N*-2-acetylaminofluorene modification on the conformation of nucleic acids. Cancer Res 34:319–327, 1974

(12) Kapuler AM, Michelson AM: The reaction of the carcinogen *N*-acetoxy-2-acetylaminofluorene with DNA and other polynucleotides and its stereochemical implications. Biochim Biophys Acta 232:436–450, 1971

(13) Kriek E, Reitsema J: Interaction of the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene with polyadenylic acid: Dependence of reactivity on conformation. Chem Biol Interact 3:397–401, 1971

(14) Weinstein IB, Grunberger D: Structural and functional changes in nucleic acids modified by chemical carcinogens. *In* Chemical Carcinogenesis, Part A (Ts'o PO, DiPaolo JA, eds). New York: Marcel Dekker, 1974, pp 217–235

(15) Grunberger D, Weinstein IB: The base displacement model: An explanation for the conformational and functional changes in nucleic acids modified by chemical carcinogens. *In* Biology of Radiation Carcinogenesis (Yuhas JM, Tennant RW, Regan JD, eds). New York: Raven Press, 1976, pp 175–187

(16) Grunberger D, Nelson JH, Cantor CR, et al: Coding and conformational properties of oligonucleotides modified with the carcinogen *N*-2-acetylaminofluorene. Proc Natl Acad Sci USA 66:488–494, 1970

(17) Nelson JH, Grunberger D, Cantor CR, et al: Modification of ribonucleic acid by chemical carcinogens. IV. Circular dichroism and proton magnetic resonance studies of oligonucleotides modified with *N*-2-acetylaminofluorene. J Mol Biol 62:331–346, 1971

(18) Grunberger D, Blobstein SH, Weinstein IB: Modification of ribonucleic acid by chemical carcinogens. VI. Effect of *N*-2-acetylaminofluorene modification of guanosine on the codon function of adjacent nucleosides in oligonucleotides. J Mol Biol 82:459–468, 1974

(19) Fuchs RP, Daune MP: Dynamic structure of DNA modified with the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. Biochemistry 13:4435–4440, 1974

(20) Fuchs RP, Lefevre J-F, Pouyet J, et al: Comparative orientation of the fluorene residue in native DNA modified by *N*-acetoxy-*N*-2-acetylaminofluorene and two 7-halogeno derivatives. Biochemistry 15:3347–3351, 1976

(21) Fuchs RP: In vitro recognition of carcinogen induced local denaturation sites in native DNA by S_1 endonuclease from *Aspergillus oryzae*. Nature 257:151–152, 1975

(22) Yamasaki H, Pulkrabek P, Grunberger D, et al: Differential excision from DNA of the C-8 and N^2 guanosine adducts of *N*-acetyl-2-aminofluorene by single-strand endonucleases. Cancer Res 37:3756–3760, 1977

(23) Yamasaki H, Leffler S, Weinstein IB: Effect of *N*-2-acetylaminofluorene modification on the structure and template activity of DNA and reconstituted chromatin. Cancer Res 37:684–691, 1977

(24) Kaplan LA, Weinstein IB: Preferential inhibition of the synthesis of 45S ribosomal RNA precursor by *N*-acetoxyacetyl-aminofluorene in rat liver epithelial cultures. Chem Biol Interact 12:99–108, 1976

(25) Harvan DJ, Hass RJ, Lieberman MW: Adduct formation between the carcinogen *N*-acetoxy-2-acetylaminofluorene and synthetic polydeoxyribonucleotides. Chem Biol Interact 17:203–210, 1977

(26) Fujimura S, Grunberger D, Carvajal G, et al: Modifications of ribonucleic acid by chemical carcinogens. Modification of *Escherichia coli* formylmethionine transfer ribonucleic acid with *N*-acetoxy-2-acetylaminofluorene. Biochemistry 11:3629–3635, 1972

(27) Pulkrabek P, Grunberger D, Weinstein IB: Effects of the ionic environment on modification of yeast tyrosine transfer ribonucleic acid with *N*-acetoxy-2-acetylaminofluorene. Biochemistry 13:2414–2419, 1974

(28) Kriek E, Spelt CE: Differential excision from DNA of the C-8 deoxyguanosine reaction products of *N*-hydroxy-2-aminofluorene and *N*-acetoxy-*N*-acetyl-2-aminofluorene by endonuclease S_1 from *Aspergillus oryzae*. Cancer Lett 7:147–154, 1979

(29) Santella RM, Kriek E, Grunberger D: Circular dichroism and proton magnetic resonance studies of dApdG modified with 2-aminofluorene and 2-acetylaminofluorene. Carcinogenesis 1:897–902, 1980

(30) Grunberger D, Weinstein IB: Modifications of ribonucleic acid by chemical carcinogens. III. Template activity of polynucleotides modified by *N*-acetoxy-2-acetylaminofluorene. J Biol Chem 246:1123–1128, 1971

(31) Troll W, Rinde E, Day P: Effect on *N*-7 and C-8 substitution of guanine in DNA on T_m, buoyant density and RNA polymerase priming. Biochim Biophys Acta 174: 211–219, 1969

(32) Millette RL, Fink LM: The effect of modification of T7 DNA by the carcinogen *N*-2-acetylaminofluorene: Termination of transcription in vitro. Biochemistry 14: 1426–1431, 1975

(33) Grunberger G, Yu F-L, Grunberger D, et al: Mechanism of *N*-hydroxy-2-acetylaminofluorene inhibition of rat

hepatic ribonucleic acid synthesis. J Biol Chem 248: 6278–6281, 1973

(*34*) Yu F-L, Grunberger D: Multiple sites of action of *N*-hydroxy-2-acetylaminofluorene in rat hepatic nuclear transcription. Cancer Res 36:3629–3633, 1976

(*35*) Adams MP, Goodman JI: RNA synthesis and RNA polymerase activity in hepatic nuclei isolated from rats fed the carcinogen 2-acetylaminofluorene. Biochem Biophys Res Commun 68:850–857, 1976

(*36*) Glazer RI, Glass LE, Menger FM: Modification of hepatic ribonucleic acid polymerase activities by *N*-hydroxy-2-acetylaminofluorene and *N*-acetoxy-2-acetylaminofluorene. Mol Pharmacol 11:36–43, 1975

(*37*) Witkin EM: Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli.* Bacteriol Rev 40:869–907, 1976

(*38*) Radman M, Villani G, Boiteux S, et al: On the mechanism and genetic control of mutagenesis induced by carcinogenic mutagens. *In* Origins of Human Cancer (Hiatt H, Watson JD, Winsten JA, eds). New York: Cold Spring Harbor Laboratory, 1977, pp 903–922

(*39*) Sarasin AR, Hanawalt PC: Carcinogens enhance survival of UV-irradiated simian virus 40 in treated monkey kidney cells: Induction of a recovery pathway? Proc Natl Acad Sci USA 75:346–350, 1978

(*40*) DasGupta UB, Summers WC: Ultraviolet reactivation of herpes simplex virus is mutagenic and inducible in mammalian cells. Proc Natl Acad Sci USA 75:2378–2381, 1978

(*41*) Day RS III, Scudiero D, Dimattina M: Excision repair by human fibroblasts of DNA damaged by r-7, t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Mutation Res 50:383–394, 1978

Structural Modification and Protein Recognition of DNA Modified by *N*-2-Fluorenylacetamide, Its 7-Iodo Derivative, and by *N*-2-Fluorenamine[1, 2]

M. P. Daune,[3] R. P. Fuchs,[3] *and* M. Leng[4]

ABSTRACT—Several experimental approaches were used to compare the structural modifications introduced by the binding to DNA of *N*-hydroxy-*N*-2-fluorenamine and of *N*-acetoxy-*N*-2-fluorenylacetamide and its 7-iodo derivative. In the three binding reactions, the major part of the substitution occurred at C-8 of guanine. Formaldehyde unwinding, hydrolysis by endonuclease S_1, linear electric dichroism, circular dichroism, use of antibodies, unwinding of covalently closed circular DNA, and binding of a tripeptide were thus successively examined. Most of the techniques showed marked differences between the different modified DNA's. However, some experiments failed to show discrimination between the induced structural alterations. These specific points are discussed. On the basis of all the available experimental evidence and after examination of the Cory-Pauling-Koltun molecular model, we proposed three models of DNA binding at the C-8 of the guanine residue with 1) the 7-iodo derivative (outside binding), 2) *N*-acetoxy-*N*-2-fluorenylacetamide (insertion–denaturation) and 3) *N*-hydroxy-*N*-2-fluorenamine (insertion without denaturation).—Natl Cancer Inst Monogr 58: 201–210, 1981.

A comparative study of the binding of *N*-AcO-2-FAA and its 7-iodo derivative *N*-AcO-7-IFAA or *N*-AcO-7-FFAA to DNA in vitro was initiated (*1*) following the observation of the noncarcinogenicity of *N*-2-IFAA as opposed to the high carcinogenic potency of the parent 2-FAA in rats (*2*).

Actually, recent experiments indicate that the iodo derivative is at least as carcinogenic in rats at the site of injection as the parent compound provided that a model, ultimate metabolite (the *N*-myristoyloxy ester) is injected (Miller JA, Miller EC: Personal communication). Moreover, when tested in the *Salmonella typhimurium* system, i.e., TA98, frameshift strain, both the N-hydroxy and the N-acetoxy derivatives of the 7-iodo compound are more mutagenic than the parent compound (*3*). Lack of metabolic activation of 2-IFAA has been suggested as an explanation (*4*). Previous studies reported from this laboratory (*1, 5, 6*) have shown a different conformational change induced locally in native DNA on the one hand by the binding of *N*-AcO-2-FAA or its 7-fluoro derivative (insertion-denaturation model) and on the other hand by the binding of *N*-AcO-7-IFAA (outside binding model). The insertion-denaturation model states that the fluorene ring is accommodated between the adjacent base pairs (*7, 8*), which results in a local denaturation of the double helix. Similar conclusions have been reached for the structure of oligonucleotides modified with *N*-AcO-2-FAA (*9, 10*). In the outside binding model, the iodofluorene residue is believed to lie along the phosphate sugar backbone of the DNA double helix (*6*); the bulky iodine atom probably prevents the insertion of the fluorene ring and, therefore, an almost nondenaturating addition product is formed (*1*).

Several experimental procedures support these 2 models: formaldehyde unwinding (*11*), endonuclease S_1 digestion (*5*), linear electric dichroism (*6*), and circular dichroism of the model compounds (*4*).

More recent experiments appear either to confirm the differences between the 2 derivatives in modification of the local structure of DNA after covalent binding or to indicate an apparent similarity of the corresponding structural perturbation.

In this paper we discuss the exact meaning of each experiment and try to present a more detailed structural picture of the modified site.

QUALITATIVE AND QUANTITATIVE DETERMINATION OF THE ADDUCTS

The 7-iodo and 7-fluoro derivatives of *N*-AcO-2-FAA showed almost the same chemical reactivity toward guanosine and native and heat-denatured DNA as did the parent compound. We (*4*) have shown that the major 2-IFAA adduct results from the binding of the carcinogen to the C-8 of guanine as with 2-FAA. A minor purine adduct was also

Abbreviations: *N*-AcO-2-FAA = *N*-acetoxy-*N*-2-fluorenylacetamide; *N*-AcO-7-IFAA = *N*-AcO-7-iodo derivative of *N*-AcO-2-FAA; *N*-AcO-7-FFAA = *N*-AcO-7-fluoro derivative of *N*-AcO-2-FAA; T_m = melting temperature; 2-FA = 2-fluorenamine.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

[2] Supported by the Délégation Générale a la Recherche Scientifique et Technique (Contract No. 76.7.1676, ACC Cancérogénèse et Pharmacologie du Cancer) and by the Institut National de la Santé et de la Recherche Médicale (AT/FA No. 28, "Mecanismes de certains effets susceptibles d'être produits chez l'homme par les constituants de la fumée du tabac").

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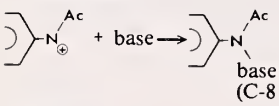
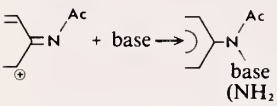
TABLE 1.—*Percentages of arylamidation and arylation*

Mechanism of reaction	DNA		Heat-denatured DNA	
	DNA-2-FAA	DNA-2-IFAA	DNA-2-FAA	DNA-2-IFAA
	2.8–5.85[a]	0.1 to 2.45[a]	3 to 4.3[a]	6
Arylamidation  base (C-8 of guanine)	84[b]	77[b]	98[b]	97[b]
Arylation  base (NH_2 of guanine or adenine)	16[c]	23[c]	2[c]	3[c]

[a] The percentage of modified bases is obtained by the ratio of C:P, where C is the concentration of [^{14}C]acetyl and P is the concentration of nucleotides.

[b] These are the relative counts in the supernatant fraction of the ethanol precipitate.

[c] These are the relative counts in the pellet fraction of the ethanol precipitate.

detected (*4*) with properties similar to the product identified by Westra et al. (*12*), i.e., 3-(deoxyguanosine-N^2-yl)-2-FAA, an N^2 adduct. Fuchs (*13*) developed a radiochemical determination of the amount of fluorene residues bound to DNA via arylamidation (C-8 adducts) or arylation (N^2 adducts). This assay (*13*) is based on the difference of stability under mildly alkaline hydrolytic conditions of the [^{14}C]-labeled N-acetyl group of the 2-FAA or 2-IFAA residues linked to the DNA by either of the above reactions.

The relative amounts of arylamidation and arylation are similar for 2-FAA and 2-IFAA in both native and heat-denatured DNA (table 1). Interestingly enough, the arylation reaction (N^2 adduct) almost always takes place with nDNA, and has a yield of about 20%. However, we want to stress that, although the N^2 and C-8 are the only 2-FAA and 2-IFAA adducts which have been characterized, other minor adducts may be formed. If such adducts exist, they will be categorized as either the arylation or the arylamidation type and, therefore, will be assayed as N^2 or C-8 adducts of guanine.

Because it has not been possible to obtain a DNA with only one type of adduct, the results of the physical, chemical, and biochemical techniques used to study the structural alterations induced in DNA provided averages of the contributions of each type of adduct. Inasmuch as 2-FAA and 2-IFAA provide almost 80% of the binding at the C-8, one can assume that the structural alterations observed are produced by this major adduct.

SURVEY OF DATA OBTAINED WITH DIFFERENT TECHNIQUES

Formaldehyde Unwinding and Melting Profile

In our first attempt to distinguish between the conformational changes induced by covalent binding to DNA of 2-FAA and 2-IFAA, we (*1*) examined the stability and dynamic structure of modified DNA. Determinations of melting profiles and T_m measurements (*8*) and kinetics studies of DNA unwinding by formaldehyde (*11*) were used concurrently.

A global destabilization of the DNA molecule is detected in the first method, which is about three times higher with DNA-2-FAA than with DNA-2-IFAA.

In the second technique, a more detailed description of the disorganized structure is obtained according to the average number of base pairs which are locally open in the vicinity of the modified guanine.

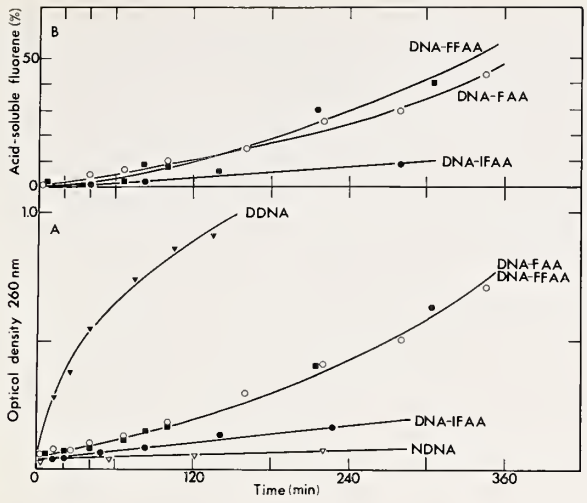
Data are summarized in table 2, and the difference of local stability between the two derivatives is striking.

Sensitivity of the Modified DNA's to a Single-Strand, Specific Endonuclease

Fuchs (*5*) has shown that the S_1 endonuclease (EC 3.1.4) from *Aspergillus oryzae* recognized and excised the local regions of denaturation induced in DNA-2-FAA (*5*). However, DNA-2-IFAA was only slightly more sensitive to endonuclease than was nDNA (text fig. 1). A more recent study (*14*) showed the preferential excision of the C-8 adduct in DNA-2-FAA and the nondenaturing type of binding of the N^2 adduct. Considerations of molecular models have recently led to similar conclusions (*15*). Therefore, the N^2 adduct most likely fits the outside binding model.

TABLE 2.—*Stability of modified DNA's: T_m and formaldehyde unwinding*

Modified DNA	T_m, degrees C	Average size of loop at 49° C No. of base pairs
DNA-2-FAA	1.15	12–12
DNA-FFAA	1.2	15–16
DNA-2-IFAA	0.4	1–2



TEXT-FIGURE 1.—Kinetics of hydrolysis of calf thymus DNA by S_1 endonuclease of *A. oryzae*. Time is given in minutes. A) Acid-soluble material was measured at 260 nm; B) Acid-soluble fluorene was determined at 305 nm. ∇ = NDNA; ▼ = DDNA; O = DNA-2-FAA; ■ = DNA-FFAA; • = DNA-2-IFAA; N = native (DNA); D = denatured (DNA).

Linear Dichroism

When native or carcinogen-modified DNA in solution encounters an electric field, orientation of the macromolecule takes place, mainly due to a large induced dipole moment that arises from the migration of counter-ions along the polyelectrolyte backbone. Chromophores, like bases or covalently bound aromatic rings, are oriented accordingly and give rise to unequal absorption of polarized light, parallel or perpendicular to the direction of orientation. An electric field pulse is applied and the response of the molecule is measured as the variation in parallel intensity of transmitted light, polarized parallel to the electric field. Under steady-state conditions and with a rod-like molecule, the relative variation of intensity is given by: D = variation in parallel intensity/intensity = $-2.3/15\ (1-3\cos^2\chi)\ KE^2 \times A$, where χ designates the angle between the transition moment corresponding to the observed wavelength and the axis of the molecule, A the absorbance at the same wavelength, K an orientation function, and E the intensity of the electrical field.

The optical anisotropy of modified DNA was measured at 265, 302, and 313 nm, respectively. At 265 nm, the contribution of the fluorene ring never exceeded 5% of the absorbance. At 302 and 313 nm, the contribution of the DNA bases was negligible. Therefore, the angle χ was readily obtained from the D_{302}:D_{265}.

In table 3, values of variation in parallel intensity/intensity are given for different values of X, the percentage of modified bases. It is clear that the χ value obtained with DNA-2-FAA (~ 80°) differs markedly from that obtained with DNA-2-IFAA. An approximate calculation, if one assumes 80% of the fluorene ring is perpendicular to the DNA axis (C-8 adducts) and 20% (N^2 adducts) is lying in the small groove, gives a value of χ equal to 77°, which is close to the observed one. The value of 65° obtained with DNA-2-IFAA disproves any classical insertion model and is in agreement with the outside binding model (*6*).

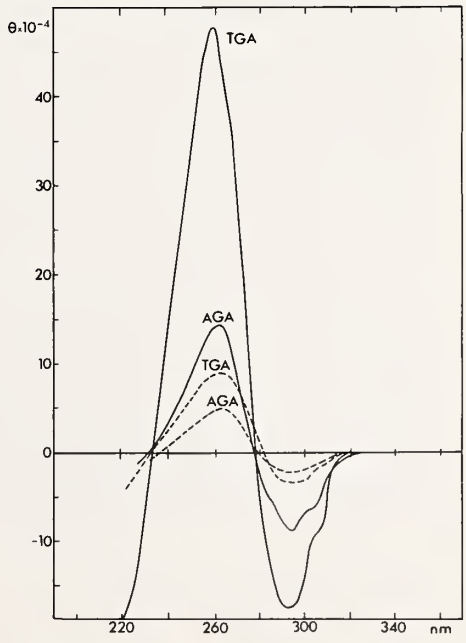
TABLE 3.—*Electric dichroism measurements on modified DNA* [a]

DNA	X	Parallel intensity/ [A] × intensity			χ (Degrees)	
		265 nm	302 nm	313 nm	302	313
DNA	0	7×10^{-2}				
DNA-2-FAA	0.7	4.54×10^{-2}	4.24×10^{-2}		81.5	80
DNA-2-IFAA	2.0	7.77×10^{-2}	3.57×10^{-2}	3.2×10^{-2}	65	64

[a] X = percentage of modified bases. Variation in parallel intensity/intensity = $-2.3/15\ (1-3\cos^2\chi)\ [A] \times AE^2$.

Circular Dichroism

When covalently attached to the DNA, the fluorene ring presents an induced optical activity in the 300- to 320-nm range in 2-FAA- and 2-IFAA-modified DNA (*1, 8*) and deoxytrinucleotides (*4*). A detailed conformational study was made by a comparison of the induced circular dichroism signal in TGA and AGA, respectively, in which the central modified guanine designates the guanine and fluoro or iodo derivatives of 2-FAA. Text-figure 2 shows the four circular dichroism spectra corresponding to the modified deoxytrinucleotide. The unmodified deoxytrinucleotide presents minimal optical activity in the 240- to 300-nm range. As previously observed with 2-FAA-modified dinucleotides (*9, 10*), dramatic changes in this form of



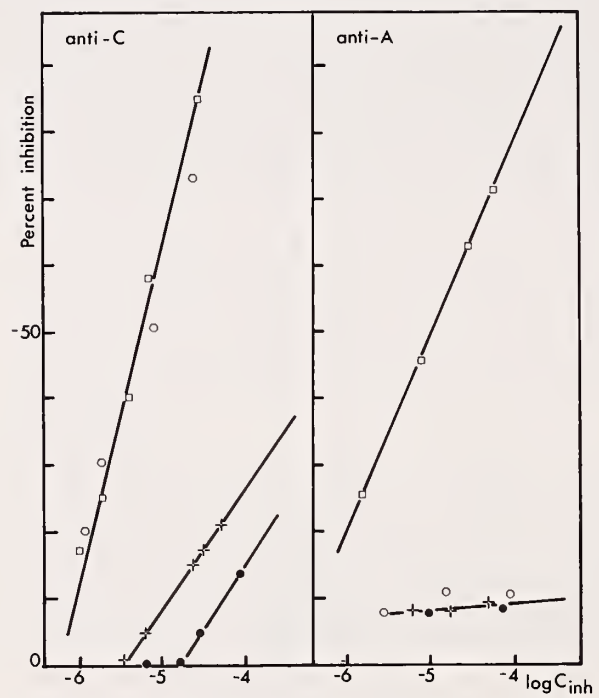
TEXT-FIGURE 2.—Circular dichroism spectra of trinucleotides TGA and AGA modified with 2-FFAA (——) and 2-IFAA (- - - -).

dichroism occur after attachment of the 2 derivatives. In the range of 300–320 nm, a comparison can be made between the modified deoxytrinucleotides and modified mononucleotides. The ratio of their respective ellipticities approximates 20 with FFAA but only about 2 with IFAA. Therefore, the iodofluorene chromophore appears to experience only the average internal field of the deoxytrinucleotide in contrast with the fluorofluorene ring which must be stacked with the adjacent bases that results in strong dipole–dipole interactions.

Use of Antinucleoside Antibodies (Anti-C and Anti-A)

The reactivity of a modified DNA with antibodies directed against adenosine and cytidine was determined by radioimmunoassay. We measured the inhibition of the precipitation of denatured [^{3}H]DNA by antinucleoside antibodies (anti-A or anti-C) as a function of the amount of modified DNA which is added to the mixture as a competitor (*16*).

In text-figure 3, the percentage of inhibition is plotted in function of the logarithm of inhibitor concentrations. With anti-A, only the denatured DNA is an efficient competitor; with anti-C, DNA-2-FAA is as efficient as dDNA, but DNA-2-IFAA is a poor competitor. The efficiency of the inhibition and, therefore, the amount of accessible cytosine is almost two orders of magnitude lower with DNA-2-IFAA than with DNA-2-FAA.



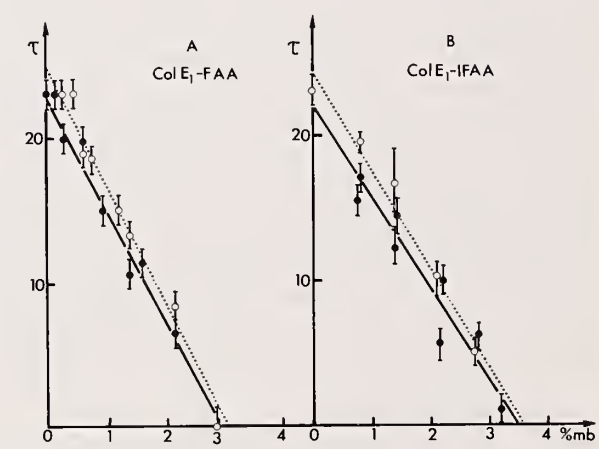
TEXT-FIGURE 3.—Competition between antibodies to cytidine (C, *left*) and to adenosine (A, *right*), |^{3}H| dDNA. □ = dDNA; ○ = nDNA-2-FAA (5.5%); + = nDNA-2-IFAA (3%); • = nDNA-2-FA (5.5%). Concentrations of inhibitor (C_{inh}) are expressed in moles of cytosine (adenosine) for DNA and in moles of fluorene-modified bases otherwise. The percentage of modified bases are indicated *in brackets*. As usual, *n* and *d* designate native and denatured DNA, respectively. All experiments were done at 4° C in 0.2 M NaCl, 5 mM Tris–HCl, pH 7.5.

From the results given by these five techniques, we concluded that 2-FAA and 2-IFAA induce two types of structural alterations when bound to DNA. The first compound and its 7-fluoro derivative are illustrative of the insertion–denaturation model [or base displacement model (*17*)], in which the fluorene ring attached to C-8 is stacked with the neighboring base pairs with the guanine left outside and with a disorganized loop induced. For 2-IFAA, the term "outside binding model" was proposed because the bound carcinogen is lying outside in one of the DNA grooves. These two models refer to the major adduct at C-8. The local conformational state of DNA at the level of the minor N^2 adduct is mostly unknown.

We must now consider more recent experimental approaches which give apparently similar results when used with DNA–2-FAA and DNA–2-IFAA.

Unwinding of Covalently Closed DNA

Covalent binding of an aromatic ring to guanine is expected to modify the conformation of the phosphodiester backbone. A sensitive probe to such subtle conformational changes was found to be the unwinding of covalently closed circular DNA, which normally presents a supercoiled structure in relation to the value of the angle between two successive base pairs. By Crick's notation (*18*), when the linking number L is kept constant, any change of the writhing number W is numerically equal but with an opposite sign to the change of the twist number T: $\Delta W = -\Delta T$. We made use of this probe to follow the changes of tertiary structure of Col E_1 DNA (form I) with the percentage of modified bases (*19*).



TEXT-FIGURE 4.—Variation of the number of superhelical turns (τ) of modified Col E_1-DNA as a function of the percentage of modified bases (mb). ●——● = indirect titration by electrophoresis in agarose gels containing increasing concentrations of ethidium bromide; ○----○ = direct titration by electrophoresis after calibration of the distance of migration. A = 2-FAA-modified DNA; B = 2-IFAA-modified DNA.

The mobility of DNA in agarose gel electrophoresis decreases when the twisted form is converted into the relaxed one. Two types of experiments were done: a direct measurement of Col E_1 untwisting and an indirect determination of the number of remaining superturns in a given sample by titration with ethidium bromide. The amount of superturns is plotted as a function of the percentage of modified bases (text-fig. 4). Whatever the technique used (direct or indirect titration), the plot is linear. Complete relaxation of Col E_1 DNA takes place at about 3% of the modified bases with DNA-2-FAA and DNA-2-IFAA. Assuming an unwinding angle of 26° for each intercalated molecule of ethidium bromide, one can estimate an unwinding angle for each residue (DNA-2-FAA or -2-IFAA) and find one at 22 ± 3° and 18 ± 3°, respectively. For DNA-2-FAA, the unwinding angle is in good agreement with an independent determination by Drinkwater et al. (*20*) who used supercoiled simian virus 40 DNA.

This relaxation process was visualized under an electron microscope after the sample was spread according to Dubochet's method (*21*). Both DNA-2-FAA and -2-IFAA appear to behave similarly (fig. 1).

Binding of the Tripeptide Lysine-Tryptophan-Lysine

This peptide bound preferentially to single-stranded regions of DNA (*22, 23*) and was used to detect the presence of open regions in double-stranded DNA after UV irradiation. Therefore, we (*24*) were tempted to use this new probe to analyze the conformational state of DNA-2-FAA and -2-IFAA (*24*). Binding involves two types of

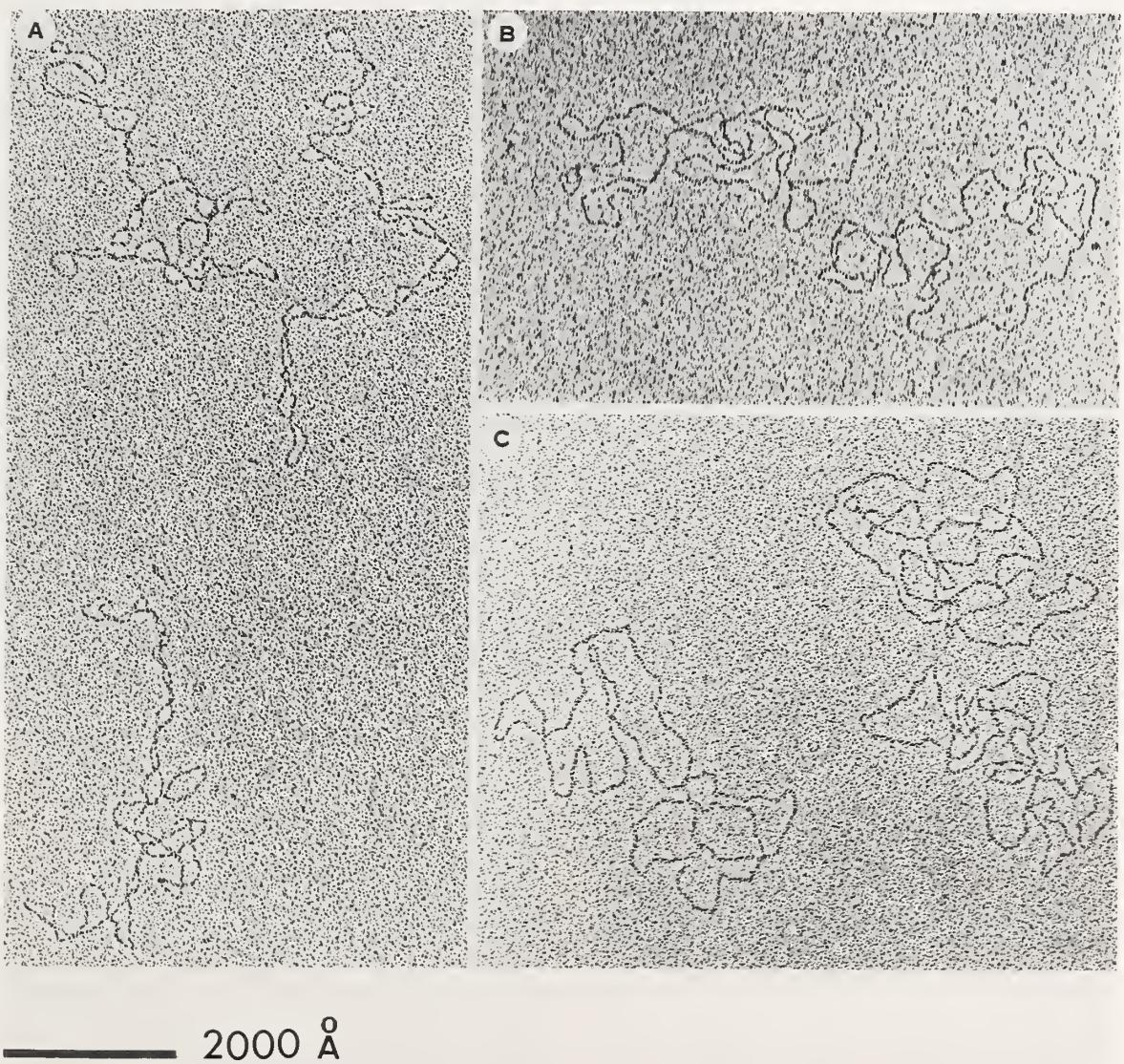


FIGURE 1.—Electron micrographs of form I Col E_1-FAA and form I Col E_1-IFAA. A) 0%, B) 2.8% 2-FAA, C) 2.8% 2-IFAA. Spreading was performed on positively charged, carbon-coated grids. The concentration of DNA is 0.5 μg/ml in 100 mM NaCl, 10 mM Tris, pH 7.4, and 0.2 mM EDTA.

complexes (*23, 25*) according to the following scheme: Nucleic acid + peptide $\rightleftharpoons$ complex I $\rightleftharpoons$ complex II.

$$K_1 \qquad K_2$$

In complex I, the tryptophan ring does not interact with bases, whereas stacking interactions take place in complex II (*26*) that lead to the fluorescence quenching of the tryptophan residue. The relative fraction of complex II is given by K_2:1 + K_2.

If the sites of modification induce locally regions of denaturation, one will expect an increase of the ratio K_2:1 + K_2 with X, the percentage of modified bases. This is indeed true, as shown in table 4. We must emphasize that: 1) The value of K_2:1 + K_2 (0.76) obtained with the highest X value (in DNA-2-FAA with 7% of modified bases) approximates that measured with denatured DNA (0.84), which is indicative of a high extent of base unpairing. 2) The values obtained for different percentages of modification with DNA-2-IFAA (only 1 value is given in table 4) do not differ from DNA-2-FAA.

From the experimental value of K_2 and the two limiting values for native and denatured DNA, it is easy to determine the amount y of peptide bound to unpaired regions and then the fraction u of unpaired regions in modified DNA. Thus u:2X (table 4) represents the average number of base pairs which are unpaired around a modified guanine. Values are decreasing when X increases, i.e., overlapping of destabilized regions occurs when X is high.

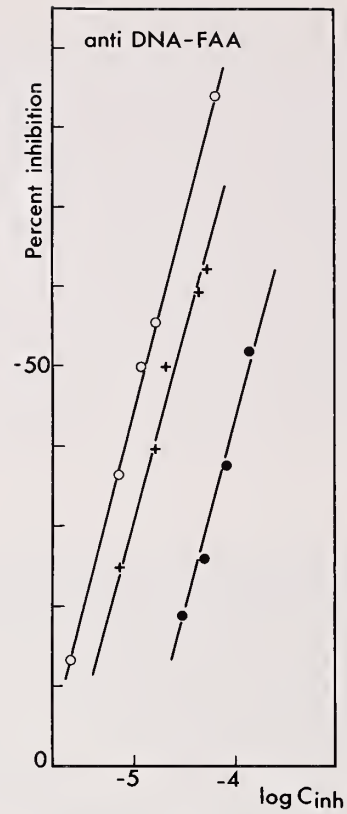
Binding of Antibodies Directed Against DNA-2-FAA and Guanosine-2-FAA Linked to Bovine Serum Albumin

Rabbits given injections of DNA-2-FAA and guanosine-2-FAA linked to bovine serum albumin triggers the production of specific antibodies which have been purified by affinity chromatography on DNA-2-FAA Sepharose columns (*27, 28*). Text-figure 5 shows that the precipitation of [^{3}H]DNA-2-FAA is slightly less inhibited by DNA-2-IFAA than by DNA-2-FAA, whereas DNA-FA is less efficient by about one order of magnitude (*16*). Similar results (not shown) were obtained with antibodies directed against guanosine-2-FAA.

After incubation of linear Col E_1 DNA-2-FAA with anti-DNA-2-FAA immunoglobulins, we could see by electron microscopy the bound antibodies (fig. 2) and determine their average number (*29*).

TABLE 4.—*LYS-TRP-LYS binding to DNA*

X		Association constants and estimation of the size of unpaired regions			
		K_2	K_2:1 + K_2	u	u:2X
nDNA	0	0.33	0.25	0	
dDNA	0	5.4	0.84	0	
DNA-2-FAA	0.41	0.75	0.43	0.09	11
DNA-2-FAA	1.05	1.06	0.515	0.15	7.5
DNA-2-FAA	2.9	1.64	0.62	0.28	5
DNA-2-FAA	7	3.12	0.76	0.62	4.5
DNA-2-IFAA	4	2.45	0.71		



TEXT-FIGURE 5.—Competition between antibodies to DNA-2-FAA, [^{3}H] DNA-2-FAA and O = nDNA-2-FAA (4%), + = nDNA-IFAA (3.6%), ● = nDNA-FA (3.6%). All other indications are as given in legend of text-figure 3.

DISCUSSION

The data obtained from most of the techniques used favor a definite difference in structure between 2-FAA and 2-IFAA bound to nDNA. However, some of the techniques seem to indicate that the structural alterations induced by both agents are similar.

A careful discussion is thus needed in which a critical appraisal of each piece of data has to be made. Instead of examining the validity of each technique to discriminate between the two adducts, we will organize the discussion around some important features of the process.

Size of the Local Defects

Without doubt, the stability of DNA-2-FAA is lower than that of DNA-2-IFAA because the observed difference in T_m is outside the range of error. A more detailed picture of the local destabilization is apparently obtained by formaldehyde unwinding. However, the estimation of the number of open base pairs around an adduct corresponds to results of an experiment made at about 10° C below the melting point because the T_m of DNA is decreased in the formaldehyde-containing reaction medium (1 M CH_2O).

Perhaps formaldehyde modifies the response of the

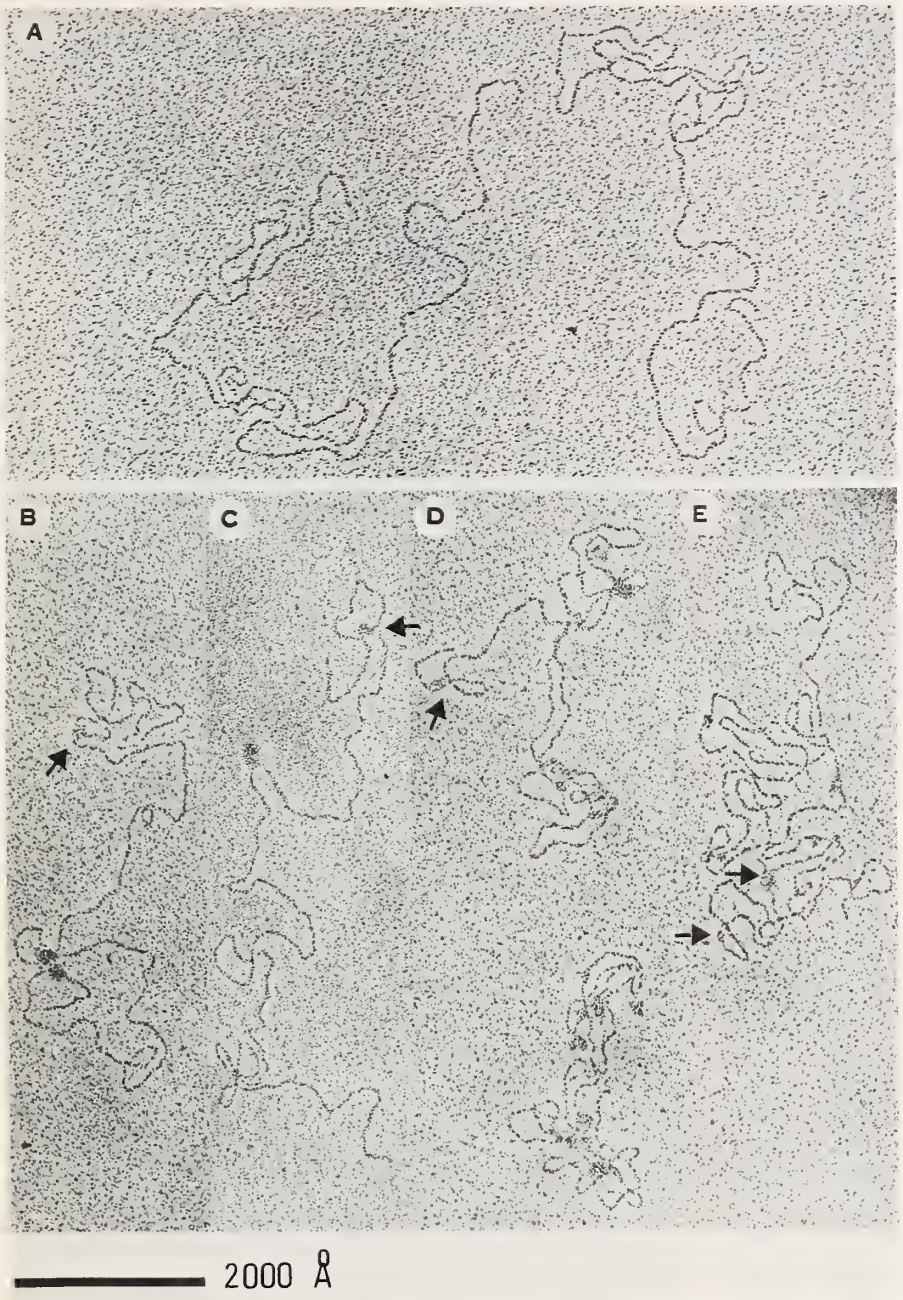


FIGURE 2.—Electron micrographs of form III Col E_1 DNA-FAA having: 0% of modified bases (control) (A); 0.07% (B, C); 0.15% (D, E). All the samples were purified on Sepharose 4B after incubation with anti-guanosine-C-8-FAA Igb at an antibody-to-antigen ratio of 10. *Arrows* show intramolecular cross-links resulting from the binding of an IgG to two antigenic determinants of a given DNA molecule. Spreading conditions are the same as in figure 1.

system and gives a number of open base pairs higher than that actually present before the action of the chemical probe. Therefore, the actual number of open base pairs around each modified guanine must definitely be lower than the values given in table 2. This is the reason for which anti-A antibodies react so poorly. At 4° C and in 0.1 M NaCl (i.e., at 78° below the T_m), the number of open base for pairs around an adduct and the number of open A residues are negligible.

Position of the Fluorene Ring

In the analysis made from the electric dichroism experiments, the determination of χ depends on the assumed angle between the helix axis and the plane of the base pair. With precisely the electric dichroism experiments that we had used, Hogan and co-workers (*30*) recently claimed that this angle is not as close to 90° as assumed in the B structure but nearer to 70°. Such a "propeller-like" DNA molecule would be in agreement with a recent theoretical model, and, on this basis, the intercalation process was critically reexamined (*31*). However, it seems that such an interpretation is not valid and relies on incorrect use of the orientation law of an apparent permanent dipole effect (*32*).

Whatever the exact value of the tilt angle, the angular position of 2-FAA differs unambiguously from that of 2-IFAA. Such a conclusion is qualitatively borne out by the analysis of the circular dichroism spectra. They were obtained with deoxytrinucleotides, and we have to assume

that the geometry of the fluorene ring relative to the bases is governed mainly by interaction with the nearest neighbor and is not greatly modified inside the DNA.

Conformation of DNA

The local opening discussed above is not the only conformational change of DNA around the binding site. As proved clearly from the disappearance of superturns inside supercoiled circular DNA, the binding of 2-FAA as well as that of 2-IFAA is accompanied by a decrease of the winding angle and likely by changes of rotation angles in the phosphodiester backbone. We must point out that similar effects are induced by the binding of the diolepoxide of benzo[*a*]pyrene, the major binding site of which is the N^2 of guanine (*20*). Recent experiments place the benzo[*a*]-pyrene adduct outside the helix (*33, 34*). Obviously, distortion of supercoiled DNA is not only induced by intercalated drugs but also probably by other local perturbations of the structure. It would be tempting to correlate this unwinding effect to the covalent linkage at N^2 of guanine which exists also in 2-FAA and 2-IFAA. However, in view of the low percentage of N^2 adducts (~20%), the unwinding angle per modified residue would reach unacceptable values (~100°).

Thus far and without further investigations, we cannot retain this similar effect of 2-FAA and 2-IFAA as being indicative of any identity of conformation at the level of the C-8 adduct.

Protein Recognition

Because the defects introduced in a DNA molecule after binding of a carcinogen, in principle, have to be repaired by specific enzymes, enzymatic recognition of such modified regions appears to be a major problem.

Three techniques related to protein–DNA interaction have been studied: digestion by endonuclease S_1, binding of a tripeptide, and binding of antibodies. Single-stranded-like regions in DNA–2-FAA or its fluoro derivative are recognized and excised by endonuclease S_1, whereas under similar conditions, DNA–2-IFAA is only slightly attacked (*5*). On the other hand, Lys-Trp-Lys, which also reacts preferentially with single-stranded regions in DNA (*22, 23*), shows similar binding to DNA–2-FAA and -2-IFAA as measured by the quenching of the fluorescence of the Trp residue (*24*). However, in regard to DNA–2-IFAA, the interpretation of Lys-Trp-Lys binding is ambiguous for the following reason: The calculation of u and u:2X (*see* table 4) relies on the fluorescence quenching of the Trp residue due to its stacking with the bases. However, the observed quenching can be accounted for by another mechanism, i.e., the energy transfer from the tryptophyl residue to the fluorene ring. A calculation of the Förster distance (*24*) shows that the quenching domain for DNA–2-IFAA is larger than with DNA–2-FAA (11.5 and 9 base pairs, respectively). Indeed, experimentally, when Lys-Trp-Lys is bound to modified denatured DNA, in which all the Trp residues are (in principle) stacked, one observes an additional quenching in the DNA–2-IFAA reaction. This additional quenching is obviously due to energy transfer.

Thus we clearly observe that an unknown fraction of the quenching, when Lys-Trp-Lys is bound to DNA–2-IFAA, is not due to the stacking of the Trp residue with the bases. The difference between DNA–2-FAA and DNA–2-IFAA in local disorganization of the helix is difficult for one to assess from the tripeptide-binding experiments. On the other hand, in the presence of a molecule with greater affinity for single-stranded regions, the conformational equilibrium can be displaced toward the opening of preexisting labile base pairing.

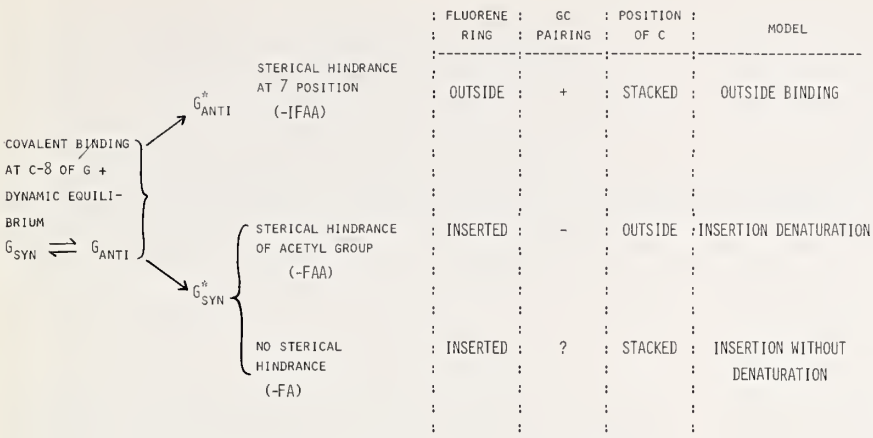
The reaction of antibodies to DNA–2–FAA (or to guanine-2–FAA) is different. Actually, these antibodies recognize dGMP–2-FAA (C-8 adduct) but not dGMP or the fluorene ring alone (*27, 28*). Their similar affinity to DNA–2–FAA and to DNA–2–IFAA can be understood, if one assumes the antigenic determinant (which is probably a small part of the guanosine–2-FAA molecule) is equally well accessible in both modified DNA's. This would also mean that 2-FAA residues are not completely buried in the DNA helix in the insertion–denaturation model. Finally, the inhibition by DNA–2-FA is less efficient (text-fig. 5) and most likely reflects the activity of the acetyl group in the antigenic determinant.

Binding Models

A comparison between the structural alteration induced in the modified DNA's was made in all the experiments described in this paper. However, DNA–2-FFAA and DNA–2-FA appeared as other materials in some of the experiments. Actually, the 7-fluoro derivative can be considered as completely like DNA–2-FAA, but this is not true for DNA–2-FA; a tentative model for the structure of DNA–2-FA will be proposed in the light of a few recent experiments.

Let us examine the approach of the electrophilic intermediate (the nitrenium ion) along the reaction path which will lead to its reaction with the C-8 of guanine. A few years ago (*35*), a role of DNA "breathing" was postulated as an explanation for the unexpected reactivity of the C-8, an atom which, in the B structure, is not easily accessible from the large groove. A transient opening of the guanine–cytosine pair (and adenine–thymine pair) is usually proposed to explain the rate of deuterium or tritium exchange of the hydrogen atom involved in base pairing (*36–38*). We now postulate that such a fast open $\rightleftharpoons$ closed dynamic equilibrium could be correlated with a *syn* $\rightleftharpoons$ *anti* conformational change which normally cannot occur inside the double-stranded structure. The covalent binding of the electrophilic reagent at C-8 can thus be conceived as a concerted reaction in which two factors would play a decisive role: 1) the rotation of a given guanine residue around the glycosylic bond, and 2) the steric properties of the approaching electrophilic reagent.

According to theoretical calculations (Pullman A: Personal communication), the approach of the fluorene ring cannot be made *in* the plane of a given guanine residue because of the repulsive positive value of the electric potential near the helix, but rather *above* or *under* this plane. In other words, a partial intercalation would be a prerequisite to the formation of the covalent bond. In view of the size



FLUORENE RING	GC PAIRING	POSITION OF C	MODEL
OUTSIDE	+	STACKED	OUTSIDE BINDING
INSERTED	-	OUTSIDE	INSERTION DENATURATION
INSERTED	?	STACKED	INSERTION WITHOUT DENATURATION

TEXT-FIGURE 6.—Schematic proposal of different modes of binding (*see* text for explanation).

of the molecule, the large groove appears to be the best "standby" region.

After examination of the different possibilities in a Cory-Pauling-Koltun molecular model and to maintain the approach of the electrophile from the large groove, we propose three binding models:

1) When a sterical hindrance exists (like a bulky substituent at the 7-position) that prevents any stacking with the adjacent bases, the fluorene ring is kept outside and lying in the groove. Due to the energy balance (mainly hydration energy), the guanine residue remains stacked in the *anti* conformation and might even be hydrogen bonded with the complementary cytosine residue. This happens with the 2-IFAA adduct at C-8, and the general features correspond to what we have called the outside binding model (text-fig. 6).

2) When the aromatic ring can be accommodated instead of guanine, the stacking of the fluorene ring with the adjacent bases is best realized when guanine adopts the *syn* conformation. However, the acetyl group hinders the complete insertion of the 2-FAA ring. Consequently, the fluorene ring takes a position in which the opposite cytosine residue is pushed outside the double helix and becomes unstacked. This is best shown by the anti-cytosine antibody-binding experiments (text-fig. 3). The insertion of this large aromatic ring instead of the guanine residue disorganizes the DNA structure locally. This is what occurs with the 2-FAA or its 7-fluoro derivative adduct at C-8; these structural alterations are described by the so-called insertion-denaturation model or the base displacement model (text-fig. 6).

3) Only limited experimental evidence is available now to characterize the structure of the 2-FA adduct. A general destabilization of the DNA molecule (decrease of T_m) was observed (*39*), but hydrolysis by endonuclease S_1 was similar to that observed with DNA-2-IFAA. The presence of a large disorganized region was ruled out.

As revealed by the anti-cytosine antibody-binding studies (*16*), this amino acid was not accessible (text-fig 3).

NOTE ADDED IN PROOF

Recently, several authors have investigated the structure of the -FA adducts both at the level of the mononucleotides and dinucleotides (*40–42*) and at the level of the DNA (*39, 43*). The data obtained suggest that the dGuo-FA adopts the anticonformation which seems to be stabilized by a hydrogen bond between the NH group of the aminofluorene moiety and the 5′-oxygen of the deoxyribose. As it stands now, we would therefore suggest that the conformation around the -FA adduct is similar to that described for the -IFAA adduct, i.e., that described by the outside binding model (text-fig. 6).

REFERENCES

(*1*) FUCHS R, DAUNE M: Physical bases of chemical carcinogenesis by *N*-2 fluorenylacetamide derivatives and analogs. FEBS Lett 34:295–298, 1973

(*2*) MORRIS HP, VELAT CA, WAGNER BP, et al: Studies of carcinogenicity in the rate of derivatives of aromatic amines related to *N*-2-fluorenylacetamide. J Natl Cancer Inst 24:149–180, 1960

(*3*) SANTELLA RM, FUCHS RP, GRUNBERGER D: Mutagenicity of 7-iodo and 7-fluoro derivatives of *N*-hydroxy and *N*-acetoxy-*N*-2-acetylaminofluorene in the *Salmonella typhimurium* assay. Mutat Res 67:85–87, 1979

(*4*) LEFÈVRE JF, FUCHS RP, DAUNE MP: Comparative study on the 7-iodo and 7-fluoro derivatives of *N*-acetoxy-*N*-2-acetylaminofluorene: Binding sites on DNA and conformational change of modified deoxytrinucleotides. Biochemistry 17:2561–2567, 1978

(*5*) FUCHS RP: In vitro recognition of carcinogen-induced local denaturation sites in native DNA by S_1 endonuclease from *Aspergillus oryzae.* Nature 257:151–152, 1975

(*6*) FUCHS RP, LEFÈVRE JF, POUYET J, et al: Comparative orientation of the fluorene residue in native DNA modified by *N*-acetoxy-*N*-2-acetylaminofluorene and two 7-halogeno derivatives. Biochemistry 15:3347–3351, 1976

(*7*) FUCHS R, DAUNE M: Changes of stability and conformation of DNA following the covalent binding of a carcinogen. FEBS Lett 14:206–208, 1971

(*8*) _____: Physical studies on DNA after covalent binding of a carcinogen. Biochemistry 11:2659–2666, 1972

(*9*) GRUNBERGER D, NELSON JH, CANTOR CR, et al: Coding and conformational properties of oligonucleotides modified with the carcinogen *N*-2-acetylaminofluorene. Proc Natl Acad Sci USA 66:488–494, 1970

(*10*) NELSON JH, GRUNBERGER D, CANTOR CR, et al: Modification of ribonucleic acid by chemical carcinogens. IV.

Circular dichroism and proton magnetic resonance studies of oligonucleotides modified with *N*-2-acetylaminofluorene. J Mol Biol 62:331–346, 1971

(*11*) Fuchs RP, Daune MP: Dynamic structure of DNA modified with the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. Biochemistry 13:4435–4440, 1974

(*12*) Westra JG, Kriek E, Hittenhausen H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149-164, 1976

(*13*) Fuchs RP: Arylamidation and arylation by the carcinogen *N*-2-fluorenylacetamide: A sensitive and rapid radiochemical assay. Anal Biochem 91:663–673, 1978

(*14*) Yamasaki H, Pulkrabek P, Grunberger D, et al: Differential excision from DNA of the C-8 and N^2 guanosine adducts of *N*-acetyl-2-aminofluorene by single strand-specific endonucleases. Cancer Res 37:3756–3760, 1977

(*15*) Beland FA: Computer-generated graphic models of the N^2-substituted deoxyguanosine adduct of 2-acetylaminofluorene and benzo [*a*] pyrene and the O^6-substituted deoxyguanosine adduct of 1-naphthylamine in the DNA double helix. Chem Biol Interact 22:329–339, 1978

(*16*) Spodheim-Maurizot M, Rio P, Leng M, et al: Discrimination by antibodies between local defects in DNA induced by 2-aminofluorene derivatives. FEBS Lett 108: 66–68, 1979

(*17*) Levine AF, Fink LM, Weinstein IB, et al: Effect of *N*-2-acetylaminofluorene modifications on the conformation of nucleic acids. Cancer Res 34:319–327, 1974

(*18*) Crick FH: Linking numbers and nucleosomes. Proc Natl Acad Sci USA 73:2639-2643, 1976

(*19*) Lang MC, Freund AM, de Murcia G, et al: Unwinding of supercoiled col E_1 DNA after covalent binding of the ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene and its 7-iodo derivative. Chem Biol Interact 28: 171-180, 1979

(*20*) Drinkwater NR, Miller JA, Miller EC, et al: Covalent intercalative binding to DNA in relation to mutagenicity of hydrocarbon epoxides and *N*-acetoxy-2-acetylaminofluorene. Cancer Res 38:3247-3255, 1978

(*21*) Dubochet J, Ducommun M, Zollinger M, et al: A new preparation method for dark-field electron microscopy of biomacromolecules. J Ultrastruct Res 35:147–167, 1971

(*22*) Toulme JJ, Charlier M, Hélène C: Specific recognition of single-stranded regions in ultraviolet-irradiated and heat-denatured DNA by tryptophan-containing peptides. Proc Natl Acad Sci USA 71:3185–3188, 1974

(*23*) Toulme JJ, Hélène C: Specific recognition of single-stranded nucleic acids. J Biol Chem 252:244–249, 1977

(*24*) Toulme F, Hélène C, Fuchs RP, et al: Binding of a tryptophan-containing peptide (Lys-Trp-Lys) to DNA modified by *N*-acetoxy-*N*-2-acetylaminofluorene. Biochemistry 19:870–875, 1980

(*25*) Brun F, Toulme JJ, Hélène C: Interactions of aromatic residues of proteins with nucleic acids. Fluorescence studies of the binding of oligopeptides containing tryptophan and tyrosine residues to polynucleotides. Biochemistry 14:558–563, 1975

(*26*) Dimicoli JL, Hélène C: Interactions of aromatic residues of proteins with nucleic acids. I. Proton magnetic resonance studies of the binding of tryptophan-containing peptides to polyadenylic acid and DNA. Biochemistry 13:714–723, 1974

(*27*) Leng M, Sage E, Fuchs RP, et al: Antibodies to DNA modified by the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. FEBS Lett 92:207–210, 1978

(*28*) Sage E, Fuchs RP, Leng M: Reactivity of the antibodies to DNA modified by the carcinogen *N*-acetoxy-*N*-acetyl-2-aminofluorene. Biochemistry 18:1328–1332, 1979

(*29*) de Murcia G, Lang MC, Freund AM, et al: Electron microscopic visualization of *N*-acetoxy-*N*-2-acetylaminofluorene binding sites in Col E_1 DNA by means of specific antibodies. Proc Natl Acad Sci USA 76:6076–6080, 1979

(*30*) Hogan M, Dattagupta N, Crothers DM: Transient electric dichroism of rod-like DNA molecules. Proc Natl Acad Sci USA 75:195–199, 1978

(*31*) _____: Transient electric dichroism studies of the structure of the DNA complex with intercalated drugs. Biochemistry 18:280–288, 1979

(*32*) Sokerov S, Weill G: Polarized fluorescence in an electric field: Comparison with other electro-optical effects for rodlike fragments of DNA and the problem of the saturation of the induced moment in polyelectrolytes. Biophys Chem 10:161–171, 1979

(*33*) Geacintov NE, Prusik T: Spectroscopic studies of carcinogen-DNA complexes. Structure of the covalent benzo[*a*]pyrene 7, 8-dihydrodiol 9, 10-epoxide DNA adducts. Proc Am Assoc Cancer Res 19:88, 1978

(*34*) Geacintov NE, Gagliano A, Ivanovic V, et al: Electric linear dichroism study of the orientation of benzo[*a*]-pyrene-7,8 dihydrodiol 9,10-oxide covalently bound to DNA. Biochemistry 17:5256–5262, 1978

(*35*) Kapuler AM, Michelson AM: The reaction of the carcinogen *N*-acetoxy-2-acetylaminofluorene with DNA and other polynucleotides and its stereochemical implication. Biochim Biophys Acta 232:436–450, 1971

(*36*) McConnell B, Von Hippel PH: Hydrogen exchange as a probe of the dynamic structure of DNA. I. General acid–base catalysis. J Mol Biol 50:297–332, 1970

(*37*) Von Hippel PH, Wong KY: Dynamic aspects of native DNA structure. Kinetics of the formaldehyde reaction with calf thymus DNA. J Mol Biol 61:587–613, 1971

(*38*) Teitelbaum H, Englander SN: Open states in native polynucleotides. II. Hydrogen exchange studies of cytosine containing double helices. J Mol Biol 92:79–92, 1975

(*39*) Spodheim-Maurizot M, Saint-Ruf G, Leng M: Conformational changes induced in DNA by in vitro reaction with *N*-hydroxy-*N*-2-aminofluorene. Nucleic Acids Res 6:1683–1694, 1979

(*40*) Leng M. Ptak M, Rio P: Conformation of acetylaminofluorene and aminofluorene modified guanosine and guanosine derivatives. Biochem Biophys Res Commun 96:1095–1102, 1980

(*41*) Santella RM, Kriek E, Grunberger D: Circular dichroism and proton magnetic resonance studies of dApdG modified with 2-aminofluorene and 2-acetylaminofluorene. Carcinogenesis 1:897–902, 1980

(*42*) Evans FE, Miller DW, Beland FA: Sensitivity of the conformation of deoxyguanosine to binding at the C-8 position by N-acetylated and unacetylated 2-aminofluorene. Carcinogenesis 1:955–959, 1980

(*43*) Spodheim-Maurizot M, Saint-Ruf G, Leng M: Antibodies to N-hydroxy-2-aminofluorene modified DNA as probes in the study of DNA reacted with derivatives of 2-acetylaminofluorene. Carcinogenesis 1:807–812, 1980

Detection and Quantitation of Acetylated and Deacetylated *N*-2-Fluorenylacetamide–DNA Adducts by Radioimmunoassay[1]

Miriam C. Poirier and Stuart H. Yuspa[2, 3]

ABSTRACT—Antibody raised in rabbits has been used for the detection of picomole quantities of the major adducts formed upon interaction of activated *N*-acetoxy-2-fluorenylacetamide (*N*-AcO-2-FAA) with DNA. By radioimmunoassay the quantitation of *N*(deoxyguanosin-8-yl)-2-FAA and *N*(deoxyguanosin-8-yl)-2-fluorenamine and discernment of the proportion of each in a mixture are possible. The antibody does not recognize the minor adduct 3-(deoxyguanosin-N^2-yl)-2-FAA, 2-FAA, or DNA and is therefore specific for the acetylated and deacetylated C-8 adducts. We used the radioimmunoassay to detect and quantitate these adducts in DNA from several types of cultured cells exposed to 10^{-5} M *N*-AcO-2-FAA. Levels of bound C-8 adducts varied between 100 and 200 fmol/μg DNA for all cells investigated. In all cells except primary rat hepatocytes, 95–97% of the C-8 adducts were in the deacetylated form, but in the rat hepatocytes, 80% of the C-8 adducts were acetylated. Our attempts to manipulate the amount and proportion of C-8 adducts bound to the DNA of primary BALB/c epidermal cells met with success in two areas. When cells were exposed to the carcinogen in the absence of serum, total binding and the percentage of acetylation were increased twofold to threefold. Also, in the presence of paraoxon, 99% of the binding and the formation of all deacetylated C-8 adducts were inhibited. We also used the radioimmunoassay to monitor repair of C-8 adducts from the DNA of BALB/c epidermal cells and normal human fibroblasts (YDF line) for 24 hours after removal of the carcinogen-containing culture medium. During this interval, the BALB/c epidermal cells and YDF cells removed approximately 40 and 50%, respectively, of the C-8 adducts from the DNA. These studies demonstrated that carcinogen–DNA adduct antibodies are useful for determining specific adducts in investigations related to aromatic amine carcinogenesis.—Natl Cancer Inst Monogr 58: 211–216, 1981.

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; RIA = radioimmunoassay(s); dG-8-FAA = *N*-(deoxyguanosin-8-yl)-2-FAA; dG-8-FA = *N*-(deoxyguanosin-8-yl)-2-fluorenamine; *N*-AcO-2-FAA = *N*-acetoxy-*N*-2-fluorenylacetamide; DMSO = dimethyl sulfoxide; CsCl = cesium chloride.

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[3] The authors thank Drs. I. B. Weinstein and S. Blobstein for synthesizing the original antigen used in these studies. The linear regression equation was established by Dr. R. Connor, and the primary rat hepatocytes were supplied by Dr. G. Williams. The technical assistance of Brigitte Former and Marc Dubin was greatly appreciated.

The metabolic pathways which lead to binding of the carcinogen 2-FAA to DNA have been extensively studied in vivo and in cultured cells and bacterial mutagenesis systems (*1–6*). Details concerning the nature of 2-FAA–DNA adducts have been described in the literature (*7–9*) as well as in other presentations of this Symposium. Our recent efforts in this laboratory have concentrated on the use of immunologic techniques, e.g., RIA, to characterize further the interaction of 2-FAA with DNA. Recently, we showed that antiserum prepared in rabbits by injection of *N*-guanosin-8-yl-2-FAA can distinguish and quantitate the acetylated and deacetylated C-8 dG adducts, dG-8-FAA and dG-8-FA, formed when 2-FAA interacts with DNA (*10*). We (*11, 12*) reported that these techniques are exquisitely sensitive (measuring adducts in the femtomole range), specific for the adducts formed (the carcinogen, DNA, and unsubstituted nucleosides are not recognized), and versatile (because a radioactive compound is not required and many samples can be assayed simultaneously). The ultimate goal of these studies is the elucidation of the biologic significance of both acetylated and deacetylated C-8 adducts with regard to cellular transformation, mutagenesis, and repair, inasmuch as approximately 90% of the total in vivo binding of 2-FAA to DNA is in the form of these dG-C-8 adducts. The data presented here will detail four aspects of this work: 1) antiserum specificity and development of the RIA, 2) quantitation of acetylated and deacetylated C-8 adducts formed in different cell types, 3) factors which alter binding and/or acetylation in BALB/c mouse epidermal cells, and 4) repair or removal of C-8 dG adducts in human fibroblasts and mouse epidermal cells.

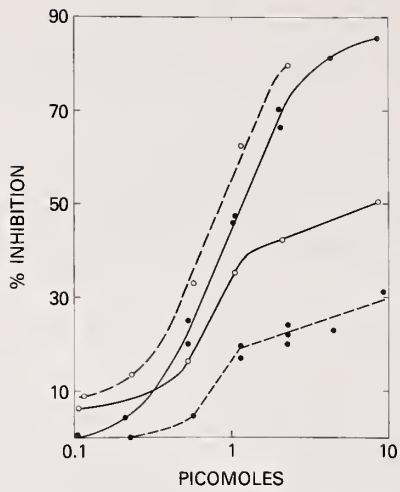
MATERIALS AND METHODS

Cell culture.—Sencar mice, bred for sensitivity to skin carcinogenesis by R. K. Boutwell from STS Rockwell × CD-1 Charles River mice (*13*), were obtained from Dr. Thomas Slaga (Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.) The BALB/c and Sencar primary mouse epidermal cells were prepared as described for BALB/c epidermal cells (*14*). Methods for maintenance of the YDF normal human fibroblast line have been described (*10*). Primary rat hepatocytes were prepared (*15*) and exposed to carcinogen in Dr. Gary M. Williams' laboratory.

Exposure to carcinogen and other chemical agents.—For these studies, semiconfluent cells were routinely grown in 150-mm dishes (Falcon Plastics, Oxnard, Calif.) and exposed to 10^{-5} M carcinogen (2-FAA or *N*-AcO-2-FAA;

obtained from the National Cancer Institute Chemical Repository, Illinois Institute of Technology Research Institute, Chicago, Ill.) in 0.4% DMSO for 1, 5, or 24 hours in medium containing 10% fetal calf serum. Other chemical agents were administered in 0.4% DMSO approximately 20 minutes before the chemical carcinogen. These include 10^{-5} M paraoxon (Aldrich Chemical Co., Milwaukee, Wisc.), 10^{-5} M ethidium bromide (Sigma Chemical Co., St. Louis, Mo.), and 10^{-3} M sodium butyrate (Sigma Chemical; butyric acid was taken to neutral pH with sodium hydroxide). Procedures for the DNA repair or carcinogen removal experiments have been published elsewhere (*10*). The DNA from harvested cells was prepared on CsCl gradients (*16*), dialyzed, and hydrolyzed with S_1 nuclease (*10*) before testing with the RIA.

Radioimmunoassay.—Immunizaton of rabbits with G-8-FAA coupled to bovine serum albumin, specificity of the antiserum obtained, preparation of labeled and unlabeled standard compounds for RIA, and the procedures for this assay were described in detail (*10–12, 17*). The procedures we used for linear regression analysis to determine the percent acetylation in mixtures containing 90–100% of the C-8 adducts in the deacetylated form are in (*17*). Procedures for the ultrasensitive enzyme-linked RIA (*18*) used in the measurement of C-8 adducts in cells exposed to paraoxon and *N*-AcO-2-FAA are described in (*19*).



TEXT-FIGURE 1.—RIA standard curves at nonequilibrium conditions (*11*) in which antiserum (diluted 1:1,000) was incubated with nonradioactive inhibitors, tracer, and goat antiserum to rabbit IgG at pH 7.4 in 0.01 M Tris HCl. [^{3}H]G-8-FAA (O) and [^{3}H]G-8-FA (●) were competed against dG-8-FAA (----) and dG-8-FA (———). The lines are as follows: O---O, [^{3}H]G-8-FAA vs. dG-8-FAA; ●——●, [^{3}H]G-8-FA vs. dG-8-FA; O——O, [^{3}H]G-8-FAA vs. dG-8-FA; ●---●, [^{3}H]G-8-FA vs. dG-8-FAA.

RESULTS

Development of the Radioimmunoassay

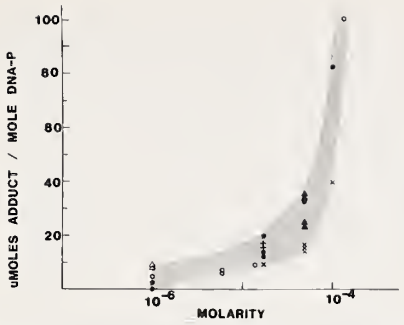
The antiserum raised against G-8-FAA does not cross-react with either 3-(deoxyguanosin-N^2-yl)-2-FAA or *N*-AcO-2-FAA alone (*11*). In early experiments, it was determined that extensive enzymatic hydrolysis of isolated DNA was required for the antibody to recognize all the C-8 adducts present on the DNA. After hydrolysis, the number of adducts on chemically modified DNA determined by UV spectrum was identical to the number of adducts determined by RIA (*11*). Furthermore, RIA profiles of hydrolyzed, chemically modified 2-FAA–DNA (1% modified) and the synthetic dG-8-FAA standard were identical (*17*). An example of an RIA standard curve in which [^{3}H]G-8-FAA was competed against unlabeled dG-8-FAA is shown in text-figure 1 (O---O). This RIA profile is the most sensitive and accurately measures as little as 0.4 pmol acetylated C-8 adduct.

Having used the acetylated C-8 adduct as the immunizing agent, we were surprised to find that the antiserum possessed considerable immunologic activity directed against the deacetylated C-8 adduct. A standared curve for [^{3}H]G-8-FA competed against unlabled dG-8-FA was similar to the standard curve for the acetylated C-8 adducts (text-fig. 1, ●——●). Evidence for the presence of distinct antibody populations was obtained in competition assays in which [^{3}H]G-8-FAA was assayed against unlabeled dG-8-FA (text-fig. 1, O——O). The saturation between 40 and 50% inhibition indicates the presence of a population of antibodies that recognize acetylated and deacetylated C-8 adducts (*10*).

Quantitation of Acetylated and Deacetylated C-8 Adducts Formed in Cultured Cells

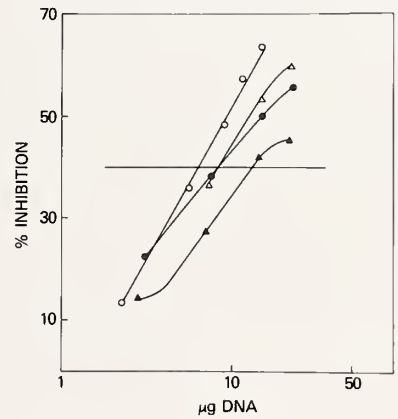
Although the studies performed with standard compounds demonstrated the capability of RIA for distinguishing and quantitating FAA–DNA adducts, our ultimate goal was detection of adducts on DNA from tissues exposed in vivo. A series of dose–response experiments were performed in which BALB/c epidermal cells were exposed in culture to increasing concentrations of *N*-AcO-2-FAA (10^{-6} to 10^{-4} M) for 1 hour (*11*). The DNA's were prepared on CsCl gradients, hydrolyzed with S_1 nuclease, and assayed by RIA. In text-figure 2, each symbol indicates samples exposed in the same experiment. The data demonstrated that increasing levels of total C-8 adducts bound to DNA with an increasing dose of carcinogen within each experiment. An indication of the variability among duplicate samples exposed to the same dose of carcinogen can be obtained by comparison of the same symbol at a particular dose. The variability among all experiments is presented by the shaded area. Carcinogen binding observed for 2×10^{-5} M *N*-AcO-2-FAA exposure, 10–20 μmol adduct/mol DNA-P, is similar to binding levels reported for confluent cultured cells exposed to [^{14}C]*N*-AcO-2-FAA under similar circumstances (*20, 21*).

To distinguish acetylated and deacetylated C-8 adducts on modified DNA's from BALB/c epidermal cells, we assayed increasing concentrations of hydrolyzed DNA in 2 RIA, one with [^{3}H]G-8-FAA and another with [^{3}H]G-8-FA as the trace compounds. If the DNA were highly acetylated, one would expect to find a competition curve similar to text-figure 1 (O---O) in the [^{3}H]G-8-FAA assay and a saturating curve similar to (●---●) in the [^{3}H]G-8-FA



TEXT-FIGURE 2.—Dose-response DNA binding data obtained by RIA from BALB/c epidermal cells treated with 10^{-6} to 10^{-4} M *N*-AcO-2-FAA for 1 hr in 0.4% DMSO. Each type of symbol represents cells exposed in 1 experiment; for example, all the (●) were treated on one occasion with 10^{-6} M, 2×10^{-5} M, 6×10^{-5} M, or 10^{-4} M *N*-AcO-2-FAA. Binding levels are expressed as micromoles adduct/mole DNA-P (*ordinate*) plotted as a function of carcinogen (*N*-AcO-2-FAA) dose (*abscissa*).

TEXT-FIGURE 3.—RIA profiles of DNA obtained from BALB/c epidermal cells exposed to 10^{-5} M *N*-AcO-2-FAA for 1 hr. The RIA procedure is similar to that described in text-figure 1 and (*11*), except that the unlabeled inhibitor is increasing concentrations of hydrolyzed FAA-DNA (micrograms DNA, *abscissa*) competed against either [^{3}H]G-8-FA (●——●) or [^{3}H]G-8-FAA (○——○).



TEXT-FIGURE 4.—RIA profiles similar to text-figure 3 in which hydrolyzed FAA-DNA's from BALB/c epidermal cells (▲——▲), YDF human fibroblasts (●——●), Sencar epidermal cells (△——△), and primary rat hepatocytes (○——○) are assayed as inhibitors against [^{3}H]G-8-FAA as the trace compound. Only the hepatocytes show complete linearity, which indicates little or no deacetylated adduct. The *horizontal line* indicates the percent inhibition at which the standard dG-8-FA is saturated against the [^{3}H]G-8-FAA in this assay.

assay. If the DNA were highly deacetylated, one would observe complete inhibition in the [^{3}H]G-8-FA assay and a saturation in the [^{3}H]G-8-FAA assay.The profiles observed can be seen in text-figure 3. The open circles indicate a saturation in the assay with [^{3}H]G-8-FAA as the trace chemical, and the closed circles indicate a complete inhibition profile with [^{3}H]G-8-FA as the trace compound. These profiles show that, in fact, most of the C-8 adduct on the BALB/c DNA is in the deacetylated form. When DNA's from 4 experiments in which BALB/c epidermal cells were exposed to *N*-AcO-2-FAA and were assayed by the linear regression procedure previously reported (*17*), the average percent acetylation value was 3.3% ± confidence limits of 0.7%.

DNA profiles in the [^{3}H]G-8-FAA assay for DNA's from BALB/c epidermal cells exposed to *N*-AcO-2-FAA, Sencar epidermal cells, YDF human fibroblasts, and primary rat hepatocytes are shown in text-figure 4. Profiles of DNA from Sencar and YDF cells showed saturation in the [^{3}H]G-8-FAA assay but were linear with higher inhibitions for the same amount of DNA in the [^{3}H]G-8-FA assay. Thus, like the DNA from BALB/c cells, these DNA's were highly deacetylated. In contrast, DNA from rat hepatocytes was linear in the assay with [^{3}H]G-8-FAA at all concentrations tested and did not inhibit in the [^{3}H]G-8-FA assay, which indicated that this DNA was highly acetylated. We could calculate from the maximum amount of hepatocyte DNA assayed with [^{3}H]G-8-FA (19 μg) that this DNA was approximately 80% acetylated.

Factors Which Alter Binding and/or C-8 Adduct Acetylation in BALB/c Epidermal Cells

When BALB/c epidemal cells were exposed to 10^{-5} M *N*-AcO-2-FAA for 1 hour in Medium 199 with 10% fetal calf serum, the levels of binding observed were 100 to 200 fmol C-8 adduct/μg DNA, and acetylation was 3.3 ± 0.7%. These are the control conditions used for the experiments shown in text-figures 2–4 and the data on the first line of table 1. If cells were exposed in Medium 199 without serum, the binding levels observed were twofold to fivefold higher than under control conditions, and competition profiles indicate the acetylated adducts increased to 8%. Thus it appears that the presence of serum in the medium lowers total binding perhaps by lowering the effective dose of carcinogen. Serum may also contribute a portion of the deacetylating activity, although the cells themselves appear to be the major determinant of acetyla-

TABLE 1.—*Variations in acetylated and deacetylated C-8 DNA adducts in mouse cells exposed to 10^{-5} M N-AcO-2-FAA under different treatment conditions*

Exposure	Total binding of C-8 adducts, % of control [a]	Percent acetylated C-8 adducts
N-AcO-2-FAA (serum)	100	3.3 ± 0.7[b]
N-AcO-2-FAA (no serum)	350 ± 100	8.0
10^{-5} M Paraoxon + *N*-AcO-2-FAA	1 ± 0.75	100.0
10^{-5} M Ethidium bromide + *N*-AcO-2-FAA	85 ± 30	2.8[b]
10^{-3} M Sodium butyrate + *N*-AcO-2-FAA	88 ± 13	3.5[b]

[a] Values are given as mean ± range of 3 experiments.

[b] Values were determined by linear regression analysis; ± confidence limits (for control only).

TABLE 2.—*RIA determination of binding and removal of C-8 adducts from the DNA of BALB/c and YDF cells* [a]

Cells	Concentration of *N*-AcO-2-FAA	fmol C-8 adduct/μg DNA[b]		Percent remaining at 24 hr
		1 hr	24 hr	
YDF	1.5×10^{-5} M	869 ± 157	432 ± 83	49.7
YDF	1.2×10^{-5} M	575 ± 73	311 ± 22	54.0
YDF	1.5×10^{-5} M	649 ± 171	319 ± 65	46.0
BALB/c	1.2×10^{-5} M	85 ± 2	50 ± 6	58.8
BALB/c	2.0×10^{-5} M	197 ± 5	127 ± 10	64.5

[a] Confluent cells were treated with *N*-AcO-2-FAA in medium containing serum. After 1-hr exposure, one-half of the dishes were harvested, whereas the other half were washed twice with phosphate-buffered saline and incubated another 23 hr in fresh carcinogen-free medium. DNA's were prepared on CsCl gradients, hydrolyzed with S_1 nuclease, and assayed by RIA.

[b] Mean ± SE from 4 to 5 replicate experiments; each experiment represents DNA extracted from 4 to 5, 150-mm dishes of cultures.

tion. Several chemical agents were tested as potential modifiers of 2-FAA binding. In an attempt at alteration of the structure of DNA at the time the cells are exposed to 2-FAA, ethidium bromide was selected because it is a known intercalator of DNA (*22*). Sodium butyrate inhibits the deacetylation of histones (*23*) and was chosen as a possible general inhibitor of deacetylation in the nucleus. Paraoxon delays the action of a microsomal deacetylase which appears to be important in the activation of 2-FAA to a mutagen (*5, 24*). All compounds were administered at doses previously shown to have no effect on cell growth or macromolecular synthesis. Table 1 demonstrates that neither ethidium bromide nor sodium butyrate altered the levels of binding or the extent of acetylated adduct formation significantly from the controls. However, paraoxon totally abolished binding as assayed in the RIA. Extremely low levels of binding (1.8–5 fmol/μg DNA) could be detected in these DNA's by ultrasensitive enzyme-linked RIA (*18*), and all of the adducts bound appeared to be acetylated because increasing the concentrations of DNA gave a linear profile against [^{3}H]G-8-FAA [similar to text-fig. 1, O --- O; (*19*)].

Repair or Removal of C-8 Adducts from the DNA of Mouse Epidermal and Human Fibroblast Cells

A particularly important application of an RIA of this type is the direct measure of adduct removal from DNA. Because repair has been extensively studied in human skin fibroblasts, and scientists from other laboratories have provided data on the removal of radioactively labeled 2-FAA adducts (*20, 21*), removal of C-8 adducts from the DNA of YDF cells [a line of normal human fibroblasts; (*10*)] was determined by RIA. The data shown in table 2 correlate well with those from other researchers and indicate that about one-half of the 2-FAA C-8 adducts were removed from the DNA of confluent YDF cells in a 24-hour repair period. Similar experiments with BALB/c epidermal cells indicated a 40% removal at 24 hour or 60% of the C-8 adducts remaining on the DNA (table 2). Inasmuch as the shape of the profiles of DNA assayed against [^{3}H]G-8-FAA in the RIA were identical at 1 hour and 24 hours (data not shown), it is likely that the acetylated and deacetylated C-8 adducts were removed at the same rate.

DISCUSSION

The specificity of the G-8-FAA antiserum and validation of the RIA have been discussed in detail elsewhere (*10–12, 17*). The procedure for distinguishing proportions of acetylated and deacetylated C-8 adducts in a mixture is based on a comparison of the profile of each DNA, in assays run with [^{3}H]G-8-FAA and [^{3}H]G-8-FA, to profiles of known standard mixtures. The percent acetylation of C-8 adducts in DNA's which are highly deacetylated (90% or above) can be determined within ± 1% from a linear regression equation (*17*). The capability of determining mixtures of other proportions with equivalent precision is still being developed; however, estimation of ± 3% acetylation can be projected on the basis of comparison with appropriate standard curves.

The degrees of acetylation and deacetylation observed in different types of cells is important for purposes of comparison with in vivo adducts in the same tissues, as well as for characterizing adducts on DNA during carcinogenesis, repair, and mutagenesis. Kriek's (*8, 25*) original observations in rat liver indicated that the largest portion (70%) of DNA-bound product (after injections of *N*-OH-2-FAA or *N*-AcO-2-FAA) was the deacetylated C-8 adduct, dG-8-FA. Our data indicate that cultured primary hepatocytes do not metabolize 2-FAA in an identical fashion with their in vivo counterpart because more than 80% of the C-8 adducts observed in these cells (exposed to either *N*-AcO-2-FAA or 2-FAA) were acetylated. Also interesting is the observation that more than 90% of FAA adducts on DNA from a number of other cells in culture (including BALB/c and Sencar epidermal cells and fibroblasts, human fibroblasts, and rat epidermal cells and fibroblasts) were deacetylated. This finding makes rat hepatocyte cultures a unique population

which could be useful for one's understanding of the regulation of deacetylation.

The mechanism by which deacetylation occurs in BALB/c epidermal cells may be partly due to factors in serum, but the major pathway appears to be a specific microsomal deacetylase in the target cells. This deacetylase is sensitive to an acetylcholinesterase inhibitor, paraoxon or diethyl-*p*-nitrophenylphosphate (*5, 24*). The dramatic reduction in binding (99% decrease) in BALB/c cells pretreated with paraoxon prior to exposure to *N*-AcO-2-FAA and the demonstration that acetylated adducts represent the residual 1% of bound material suggest that deacetylation is a critical step in the pathway which leads to DNA binding. Our capability to manipulate the proportions of acetylated and deacetylated adducts formed on DNA after *N*-AcO-2-FAA exposure in cell culture should facilitate studies designed for the elucidation of the importance of each type of adduct for mutagenesis and carcinogenesis.

Data from other scientists have demonstrated a rapid and efficient removal of FAA adducts from the DNA of human fibroblasts [approximately 50% in 24 hr; (*20 21*)]. A similar figure has been observed in human dermal fibroblasts with the RIA. Repair of 2-FAA damage in BALB/c epidermal cells has been less extensively studied, although repair replication has been observed (Dubin MA: Unpublished observations) with the use of 5-bromodeoxyuridine gradients (*26*). Data from the RIA indicate that BALB/c epidermal cells remove C-8 adducts almost as efficiently as human fibroblasts and both acetylated and deacetylated adducts are removed at the same rate.

The potential of this technique for monitoring specific adducts on DNA is still being explored. Data in this presentation demonstrated that scientists can use the RIA to distinguish between acetylated and deacetylated deoxyguanosine C-8 adducts formed in different cells and follow the removal of C-8 adducts that presumably occurs as the result of repair processes. In addition, when cells are exposed to *N*-AcO-2-FAA under conditions which alter the levels of binding and/or ratio of acetylation to deacetylation, these differences can be determined by the RIA. We anticipate that this immunologic approach will be useful in a variety of studies related to aromatic amine carcinogenesis, and the antiserum against 2-FAA-G will be the prototype for the development of many carcinogen-nucleotide antibodies.

REFERENCES

(*1*) Miller JA, Miller EC: The metabolic activation of carcinogenic aromatic amines and amides. Prog Exp Tumor Res 11:273-301, 1969

(*2*) Thorgeirsson SS, Wirth PJ, Nelson WL, et al: Genetic regulation of metabolism and mutagenicity of 2-acetylaminofluorene and related compounds in mice. *In* Origins of Human Cancer, Cold Spring Harbor Conferences on Cell Proliferation (Hiatt HH, Watson JD, Winston JA, eds), vol IV. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1977, pp 869-886

(*3*) Weisburger JH, Weisburger EK: Biochemical formation and pharmacological, toxicological, and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol Rev 25:1-66, 1973

(*4*) Maher VM, Miller EC, Miller JA, et al: Mutations and decreases in density of transforming DNA produced by derivatives of the carcinogens 2-acetylaminofluorene and *N*-methyl-4-aminoazabenzene. Mol Pharmacol 4:411-426, 1968

(*5*) Schut HA, Wirth PJ, Thorgiersson SS: Mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system: The role of deacetylation by liver and kidney fractions in mouse and rat. Mol Pharmacol 14:682-692, 1978

(*6*) Stout DL, Becker FF: Metabolism of 2-aminofluorene and 2-acetylaminofluorene to mutagens by rat hepatocyte nuclei. Cancer Res 39:1168-1173, 1979

(*7*) Miller JA, Miller EC: Activation of carcinogenic aromatic amines and amides by N-hydroxylation in vivo. *In* Carcinogenesis: A Broad Critique (A Collection of Papers Presented at the Twentieth Annual Symposium on Fundamental Cancer Research). Baltimore: Williams & Wilkins, 1967, pp 397-420

(*8*) Kriek E: Carcinogenesis by aromatic amines. Biochim Biophys Acta 355:177-203, 1974

(*9*) Westra JG, Kriek E, Hittenhausen H: Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA in vivo. Chem Biol Interact 15:149-164, 1976

(*10*) Poirier MC, Dubin MA, Yuspa SH: Formation and removal of specific acetylaminofluorene-DNA adducts in mouse and human cells measured by radioimmunoassay. Cancer Res 39:1377-1381, 1979

(*11*) Poirier MC, Yuspa SH, Weinstein IB, et al: Detection of carcinogen-DNA adducts by radioimmunoassay. Nature 270:186-188, 1977

(*12*) Poirier MC: Measurement of the formation and removal of DNA adducts of *N*-acetoxy-2-acetylaminofluorene. *In* Techniques in DNA Repair (Friedberg EC, Hanawalt PC, eds), vol 1. New York: Marcel Dekker, 1981, pp 143-153

(*13*) Boutwell RK: Some biological aspects of skin carcinogenesis. Prog Exp Tumor Res 4:207-250, 1964

(*14*) Yuspa SH, Harris CC: Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro. Exp Cell Res 86:95-105, 1974

(*15*) Williams GM: Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res 37:1845-1851, 1977

(*16*) Lieberman MW, Poirier MC: Deoxyribonucleoside incorporation during DNA repair of carcinogen-induced damage in human diploid fibroblasts. Cancer Res 33:2097-2103, 1973

(*17*) Poirier MC, Connor RJ: A radioimmunoassay for 2-acetylaminofluorene-DNA adducts. *In* Immunochemical Techniques (Van Vunakis H, Langone J, eds), vol 2. New York: Academic Press, 1981

(*18*) Harris CC, Yolken RH, Krokan H, et al: Ultrasensitive enzymatic radioimmunoassay: Application to detection of cholera toxin and rotavirus. Proc Natl Acad Sci USA 76:5336-5339, 1979

(*19*) Hsu IC, Poirier MC, Yuspa SH, et al: Ultrasensitive enzymatic radioimmunoassay (USERIA) detects femtomoles of acetylaminofluorene-DNA adducts. Carcinogenesis 1:455-458, 1980

(*20*) Amacher DE, Lieberman MW: Removal of acetylaminofluorene from the DNA of control and repair-deficient human fibroblasts. Biochem Biophys Res Commun 74:285-290, 1977

(*21*) Cerutti PA: Repairable damage in DNA. *In* DNA Repair Mechanisms: ICN-UCLA Symposia on Molecular and

Cellular Biology (Hanawalt PC, Friedberg EC, Fox CF, eds), vol IX. New York: Academic Press, 1978, pp 1–14

(*22*) LePecq JB Paoletti C: A fluorescent complex between ethidium bromide and nucleic acids. J Mol Biol 27:87–106, 1967

(*23*) Candido EP, Reeves R, Davie JR: Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14:105–113, 1978

(*24*) Irving CC: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26:1390–1396, 1966

(*25*) Kriek E: Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA in vivo. Cancer Res 32:2042–2048, 1972

(*26*) Bowden GT, Yuspa SH: Repair of daughter strand gaps in nascent DNA from mouse epidermal cells treated with dihydrodiol epoxide derivatives of benzo[*a*]pyrene. Biochim Biophys Acta 565:67–83, 1979

Repair of DNA Damage Induced in Human Fibroblasts by N-Substituted Aryl Compounds[1, 2]

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ABSTRACT—The cytotoxicity of 4 structurally related direct-acting carcinogens, i.e., *N*-acetyloxy-*N*-[1,1′-biphenyl]-4-yl-acetamide, *N*-acetoxy-2-fluorenylacetamide, *N*-acetyloxy-*N*-2-phenanthrenylacetamide, and *N*-acetoxy-4-acetylaminostilbene, was compared in diploid fibroblasts derived from normal persons and in excision repair-deficient xeroderma pigmentosum cells (XP12BE). All four proved significantly more cytotoxic to the XP cells than to the normal cells. At equicytotoxic levels, substantially more residues were initally bound to the DNA of the normal than to the XP cells, which indicated that normal cells removed a large percentage of the DNA-bound residues before these could result in cell death. The ability of these cell strains to remove DNA-bound residues, to incorporate thymidine into parental strands of DNA during repair replication, and to recover from potentially lethal damage if held in a density-inhibited, nonreplicating state was compared as a function of time. During the 6 days that the normal cells were held in the confluent G_0 state, they gradually recovered from the potentially lethal effects of these carcinogens and did so at a rate comparable to their rate of removal of DNA-bound residues and of carcinogen-induced repair replication. In contrast, the XP12BE cells showed no evidence of recovery and were virtually incapable of removing DNA adducts or of repair replication. *N*-Acetoxy-4-acetylaminostilbene proved the most cytotoxic of the 4 compounds as a function both of the concentration administered and of the number of residues initially bound to both cells strains and remaining unexcised in the XP12BE cells. The rate of excision of residues from the DNA of normal cells was phenanthrene > fluorene = biphenyl > stilbene. When the normal human cells held in confluence were assayed for removal of potentially *mutagenic* lesions induced by *N*-acetoxy-fluorenylacetamide, they exhibited a recovery correlated with removal of the cytotoxic lesions.—Natl Cancer Inst Monogr 58: 217–222, 1981.

Cellular DNA repair enzymes can moderate damage induced in DNA by physical and chemical agents (*1*). Results of a series of comparative studies with diploid human fibroblasts derived from normal individuals or patients with XP that differ in their rate of excision of DNA damage induced by UV irradiation indicate that the percent survival of the cloning ability of these cells and the frequency of UV-induced mutations to 8-azaguanine or 6-thioguanine resistance reflects their rate of removal of potentially lethal and mutagenic damage (*2, 3*). Cells with virtually no capacity for excision repair (*4*) are exceptionally sensitive to the killing action and mutagenicity of UV, and cells with an intermediate rate of repair (*5*) exhibit intermediate sensitivity (*2, 6*), whereas those with rapid rates of excision are resistant to low doses of UV (*2*). When excision repair-proficient human cells are prevented from replicating by being held in a density-inhibited state but allowed to repair DNA damage, they exhibit a gradual recovery from the potentially cytotoxic and mutagenic effects of UV (*2, 3, 7*). No recovery takes place in the XP cells which are virtually incapable of excision repair. The rate of recovery in the repair-proficient cells is directly correlated with the initial rate of UV-induced repair replication and indirectly with the initial rate of excision of thymine-containing dimers (*3*).

We recently extended these kinds of studies to human cells exposed to reactive derivatives of a series of aromatic amide (*8–10*) or polycyclic aromatic hydrocarbon (*11–13*) carcinogens. Using radioactive-labeled compounds, we have shown that the rate of recovery from potentially cytotoxic and/or mutagenic lesions induced by these agents is directly related to the rate of excision of carcinogen residues covalently bound to cellular DNA. In this report, we compare the rate of excision of DNA-bound residues of *N*-AcO-BPAA, *N*-AcO-2-FAA, *N*-AcO-PhAA, and *N*-AcO-AAS. From our results, we concluded that the rate of excision repair determines the cytotoxic (and mutagenic) effect of exposure of human cells to these carcinogens.

Abbreviations: XP = xeroderma pigmentosum; *N*-AcO-BPAA = *N*-acetyloxy-*N*-[1,1′-biphenyl]-4-yl-acetamide; *N*-AcO-2-FAA = *N*-acetoxy-2-fluorenylacetamide; *N*-AcO-PhAA = *N*-(acetyloxy)-*N*-2-phenanthrenylacetamide; *N*-AcO-AAS = *N*-acetoxy-4-acetylaminostilbene; [^{3}H]dThd = tritiated thymidine; NF = normal fibroblasts (human); BrdUrd = 5-bromodeoxyuridine; CsCl = cesium chloride.

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MATERIALS AND METHODS

Carcinogenic compounds.—Radioactive *N*-AcO-2-FAA (0.724 Ci/mmol), *N*-AcO-PhAA (1.72 Ci/mmol), *N*-AcO-BPAA (1.18 Ci/mmol), and *N*-AcO-AAS (0.296 Ci/mmol), ring-labeled with ^3H, were prepared by Dr. John D. Scribner as described in (*9*) and stored in benzene/ethanol (2:1) at −20° C to avoid autoradiolysis. Just prior to use, the labeled compounds were reduced to dryness under a stream of nitrogen gas and redissolved in anhydrous ethanol.

Cell cultures and media.—The NF were derived from foreskin material; XP12BE fibroblasts, complementation group A, were obtained from the American Type Culture Collection (Rockville, Md.). Culture medium was Ham's F10 lacking hypoxanthine, or Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) and 50 μg gentamycin/ml (Schering Corporation, Kenilworth, N.J.). The medium devised for measuring the rate of excision repair synthesis (*3*) includes 25 μM BrdUrd (Sigma Chemical Co., St. Louis, Mo.), 0.1 μM aminopterin (Sigma), 25 μM hypoxanthine (Calbiochem, San Diego, Calif.), and 15 μCi/ml [^3H]dThd [Schwarz/Mann, Orangeburg, N.Y. (50 Ci/mmol)]. The use of aminopterin, which forces the cells to rely on the exogenous source of purines and pyrimidines, allows us to control the specific activity of the intracellular nucleotide pools, whereas BrdUrd permits discrimination between newly synthesized daughter DNA strands and preexisting parental DNA.

Cytotoxicity as a function of the number of carcinogen residues bound to DNA.—The percent survival of colony-forming ability was assayed under two conditions. Exponentially growing cells were trypsinized and plated at cloning densities and allowed 12 to 16 hours for attachment. The culture medium was replaced with serum-free medium containing carcinogen which had been dissolved in anhydrous ethanol just prior to use. After 3 hours, the medium which contained a carcinogen was removed; the cells were washed, refed with fresh culture medium, and allowed to form macroscopic colonies in situ. Alternatively, cells were grown to confluence, maintained in the confluent state for 3 days without refeeding, exposed for 3 hours to labeled carcinogen as described, and then washed, trypsinized, suspended in fresh culture medium, pooled, and a portion plated at cloning densities and allowed to form colonies to determine percent survival. We used the remaining portion ($\sim 15 \times 10^6$ cells/assay) to obtain the number of carcinogen residues convalently bound to DNA.

Determination of the number of carcinogen residues covalently bound to DNA.—The procedures (*9*) we used are as follows: Cells were lysed and the DNA was extracted and centrifuged to equilibrium in CsCl. The peak fractions were pooled and the number of carcinogen residues bound per 10^6 DNA nucleotides was determined from the A_{260} profile and the specific radioactivity. Each carcinogen binding value for a specific radioactivity represents the average of at least 3 determinations from approximately 15×10^6 cells.

Rate of removal of covalently bound residues from cellular DNA.—Cells were grown to confluence, exposed to radioactive-labeled carcinogens as described and then incubated in complete repair medium but with nonradioactive dThd substituted for the [^3H]-labeled. Immediately after exposure to carcinogens or at specified intervals, cells were harvested by trypsinization, the DNA was extracted, and the parental DNA was isolated by neutral CsCl equilibrium centrifugation and analyzed for the number of carcinogen residues bound as determined from the optical absorbance and specific radioactivity.

Rate of DNA excision repair synthesis induced by these carcinogens.—Cells were grown to confluence, preincubated for 2 hours in repair medium lacking radioactive label, washed, and exposed to the respective carcinogen in repair medium but without serum. After a 3-hour exposure, the medium containing a carcinogen was replaced with complete repair medium and the treated and untreated control cultures were allowed various periods up to 6 days for excision repair to occur. At the conclusion of each repair interval, DNA was extracted, the parental DNA was isolated by two equilibrium centrifugations in alkaline CsCl, and its specific radioactivity was determined.

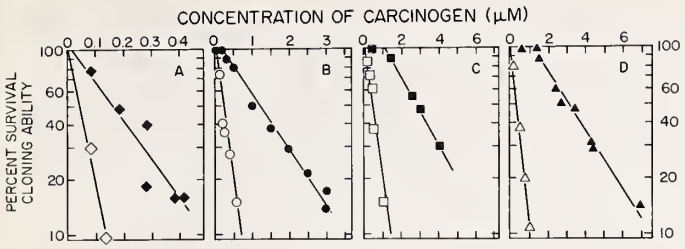
Rate of recovery of cells from potentially lethal damage.—Cultures of normal XP cells were grown to confluence, maintained for at least 3 days without refeeding, and exposed to the carcinogen for 3 hours in serum-free medium. The medium containing the carcinogen was removed and replaced with the original "spent" medium (i.e., depleted of mitogens) to prevent replication but to allow excision repair to occur (*2, 3, 9, 13*). One series of cultures was trypsinized immediately and assayed for percent survival; the rest were maintained in confluence for various intervals before being assayed for survival. During these periods, they were fed daily with spent medium.

RESULTS

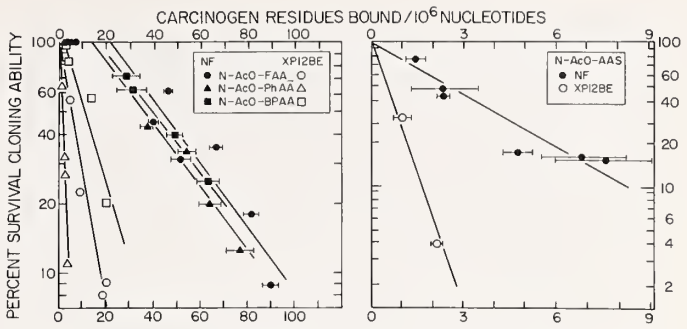
Comparison of the Cytotoxicity of the Compounds as a Function of Concentration Administered

We compared the cytotoxic effect of the N-acetoxy derivatives of the four aromatic amides in NF and XP12BE cells exposed in situ. As expected from our earlier studies (*14*), the results (text-fig. 1) indicated that the XP12BE cells are significantly more sensitive than NF to the killing action of these reactive carcinogens. In addition, examination of the cytotoxic effect at low doses showed that there was a distinct shoulder on three of the NF survival curves (1A, B, and C) that was absent in those of the XP cells. If we assumed that when the two cell populations are exposed to equal concentrations of these agents, approximately the same number of carcinogen residues are initially bound to their DNA, the results in text-figure 1 would suggest that NF possessed an excision repair system(s) capable of removing potentially lethal DNA damage induced by these compounds and that the system was defective in XP12BE cells.

Although the 4 compounds did not differ significantly if



TEXT-FIGURE 1.—Comparison of the cytotoxic effect of the N-acetoxy esters in NF and XP12BE cells as a function of concentration. Cells were plated, allowed to attach, exposed to carcinogen for 3 hr, refed with fresh culture medium, and allowed to form macroscopic colonies. Lines were fitted by eye. Each *symbol* represents percent survival averaged from 7 to 10 duplicate dishes. A) *N*-AcO-AAS; B) *N*-AcO-FAA; C) *N*-AcO-BPAA; D) *N*-AcO-PhAA.



TEXT-FIGURE 2.—Comparison of cytotoxic effect of N-acetoxy esters in NF and XP12BE cells as a function of the number of carcinogen residues initially bound to cellular DNA. Confluent cultures were exposed to radioactive-labeled carcinogen for 3 hr and then assayed, as described in "Materials and Methods," for percent survival and extent of carcinogen binding to cellular DNA. Lines for cytotoxic doses were determined by the method of least squares. *Note* the difference in the scales between the two panels.

one merely compared the ratio of the slopes of the exponential portion of the two survival curves (that of XP12BE was threefold to fourfold steeper than the NF curve), these survival curves could be analyzed in another way, i.e., by a comparison of the percent survival of the NF cells at the concentration of each carcinogen which gives a particular survival in the XP12BE strain. For example, when the survival of XP12BE was reduced to 10%, the corresponding survival of NF cells was approximately 50% for *N*-AcO-AAS, about 70% for *N*-AcO-2-FAA and 85% for *N*-AcO-BPAA, and approximately 100% for *N*-AcO-PhAA. From the results we obtained with UV irradiation (*2, 3*) that were discussed above, these differences in percent survival of the NF could be the result of differences in rate of removal of potentially cytotoxic DNA lesions caused by the respective agents. If so, then the predicted rate of removal should be *N*-AcO-PhAA > *N*-AcO-BPAA > *N*-AcO-2-FAA > *N*-AcO-AAS. As described below, this prediction was confirmed by the results of our biochemical studies (*9, 10*) on the rate of repair replication and of removal of radioactive-labeled residues.

Comparison of Carcinogen Residues Initially Bound to DNA With the Cytotoxic Effect

If the differences in survival between the NF and XP12BE cells reflected differences in their rates of DNA excision repair, the number of carcinogen residues *initially* bound to DNA of excision-proficient NF cells should have been significantly higher than in the excision-deficient XP cells at doses which result in *equal* survival. Text-figure 2 illustrates a comparison of the survival of the two cell strains as a function of the number of carcinogen residues initially bound to DNA, i.e., at the end of the 3-hour carcinogen incubation period when the cells were trypsinized and plated for survival. It is clear from these data that 1) DNA-bound residues of *N*-AcO-AAS were much more cytotoxic to the NF cells than were residues of the other 3 carcinogens; 2) NF cells attained 100% survival of colony-forming ability despite having had about 15 *N*-AcO-BPAA, *N*-AcO-2-FAA, or *N*-AcO-PhAA residues initially bound per 10^6 DNA nucleotides but could not do so with even 1 residue of *N*-AcO-AAS per 10^6; and 3) for all 4 compounds, XP12BE cells were killed by many fewer initially bound residues than were required to kill NF cells. For the purposes of comparison, the data of text-figure 2 showing the relationships between the 4 carcinogens in each strain are summarized in table 1 with similar data derived from cells exposed to UV irradiation or 2 reactive derivatives of benzo[*a*]pyrene.

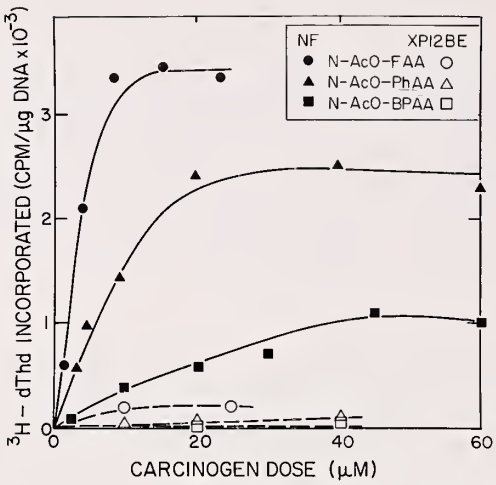
TABLE 1.—*Comparison of cell strains for number of initial DNA lesions/mean lethal event*

Carcinogenic agent	Approximate No. of residues initially bound (or dimers)/10^6 nucleotides at D_{37}	
	XP12BE	NF
N-AcO-BPAA	15	53
N-AcO-2-FAA	8	51
N-AcO-PhAA	3	46
N-AcO-AAS	1	4
Benzo[*a*]pyrene-4,5-oxide[a]	2	8
Benzo[*a*]pyrene-7,8-diol,9,10-epoxide (anti)[a]	2	8
UV-induced pyrimidine dimers[b]	3	46

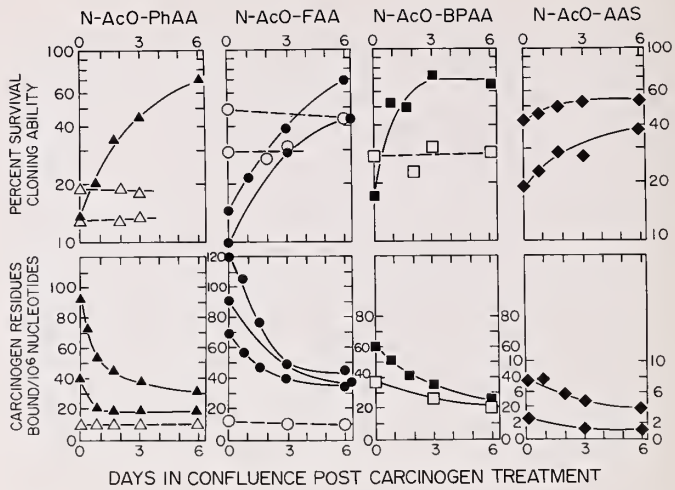
[a] Data are taken from (*13*).
[b] Data are estimated from those in (*2, 3*).

Repair Synthesis Induced in Normal and XP12BE Cells by 3 Carcinogens

DNA damage induced by UV radiation and by *N*-AcO-2-FAA elicited repair synthesis in normal human cells but not in certain XP cells (*14–17*). To determine whether a similar process occurs in response to damage produced by *N*-AcO-BPAA and *N*-AcO-PhAA, we compared the amount of repair synthesis induced in normal and XP12BE cells during the first 36 hours following exposure to



TEXT-FIGURE 3.—Comparison of NF and XP12BE cells for amount of repair replication induced by *N*-AcO-BPAA, *N*-AcO-2-FAA, or *N*-AcO-PhAA as a function of concentration. Confluent cultures were exposed to chemicals for 3 hr and then allowed to repair in medium containing BrdUrd and [^{3}H]dThd for 36 hr. Nondensity-labeled DNA (parental strands) extracted from these cultures was isolated by two successive bandings on alkaline CsCl equilibrium gradients and its specific radioactivity determined.



TEXT-FIGURE 4.—Comparison of the rates of biological recovery and removal of DNA-bound carcinogen residues by NF (*closed circles*) and XP12BE cells (*open circles*) treated in confluence with the 4 N-acetoxy esters. *See* text-figure 2 for approximate concentrations used to obtain the initial survival levels in the 2 strains. Lines fitted by eye.

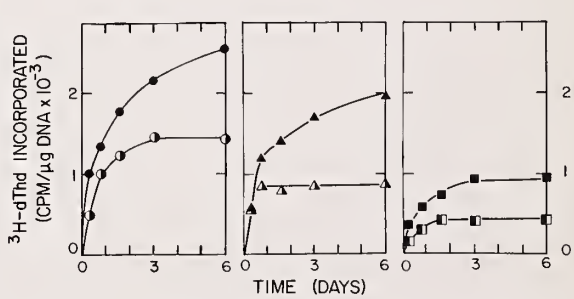
various doses of *N*-AcO-2-FAA, *N*-AcO-BPAA, or *N*-AcO-PhAA (text-fig. 3). Each carcinogen elicited a dose-dependent increase in repair synthesis in NF but little or no repair synthesis in XP12BE cells (*9*).

Comparison of the Rate of Removal of Bound Carcinogen Residues and Recovery of Cells From Potentially Lethal Effects of These Agents

Cultures of NF and XP12BE cells, inhibited from replicating by being grown to confluence, were treated with radioactive-labeled carcinogens and assayed either immediately or at various times posttreatment for the number of residues covalently bound to DNA and/or for percent survival (text-fig. 4). Several facts are evident from these comparative data: 1) The XP12BE cells were virtually incapable of removing DNA-bound residues and showed no evidence of recovery from the potentially cytotoxic effects of any of these aromatic amide derivatives; 2) the rate of recovery of the NF cells from these agents was significantly slower than from UV radiation-induced cytotoxicity (*2, 3*); 3) The rate of recovery of the NF cells was closely correlated with their rate of removal of DNA-bound residues; 4) as predicted from text-figures 1 and 2, the rate of removal of *N*-AcO-PhAA-induced residues was faster than the removal of those of *N*-AcO-BPAA or *N*-AcO-2-FAA, whereas the rate of *N*-AcO-AAS residues was much slower. However, one must take into account that during these biologic recovery experiments, the initial number of *N*-AcO-AAS-induced DNA residues was, by necessity, much lower than that of the other 3 agents because recovery could only be measured at biologically meaningful doses (text-fig. 2).

Comparison of the Initial Rates of Repair Replication Induced in Normal Cells

The data of text-figure 5 compare the amount of repair replication induced in density inhibited, nonreplicating NF cultures by 3 of these agents as a function of time posttreatment. The initial rate induced by *N*-AcO-PhAA was faster than that by *N*-AcO-2-FAA or *N*-AcO-BPAA, which agreed with the initial rates of removal of DNA residues. Inasmuch as previous studies showed that the rate of repair replication depended on the initial number of lesions present in DNA, no attempt was made to compare the rate of repair replication induced by *N*-AcO-AAS, for the reason stated above.



TEXT-FIGURE 5.—Comparison of the rate of repair replication induced in confluent cultures of NF by *N*-AcO-BPAA, *N*-AcO-2-FAA, or *N*-AcO-PhAA. Concentrations were chosen to give survival levels of 10 to 20% (*closed symbols*) or 30 to 40% (*half-closed symbols*).

DISCUSSION

The fact that human NF can excise residues of *N*-AcO-BPAA, *N*-AcO-PhAA, and *N*-AcO-AAS from their DNA, whereas XP12BE cells are virtually incapable of such excision repair explains why the latter cells are so much more sensitive to the cytotoxic action of these compounds. The data presented in this report show that the percent survival of the NF is directly correlated with their rate of removal of residues from their DNA. For example, NF originally exposed to 3.5 μM *N*-AcO-2-FAA exhibited an initial binding of approximately 90 residues/10^6 nucleotides and about 10% survival. However, after 3 days in the density-inhibited, nonreplicating state, the number of residues per 10^6 was reduced to approximately 50. What is most significant is that the percent survival of the cells harvested at that time (~30%) was almost identical to the survival of cells originally exposed to about 1.5 μM *N*-AcO-2-FAA, a concentration that resulted in 50 residues/10^6 nucleotides. These results indicate that the cytotoxic effect of treatment of the cells with *N*-AcO-2-FAA held in confluence for up to 3 days is negligible, and the cell killing observed when the cells are released and plated at lower densities reflects mainly DNA lesions remaining unexcised at the time of the harvesting of the population. This conclusion is supported by the fact that the XP12BE cells, which were virtually incapable of excising any carcinogen residues from their DNA during the time in confluence, failed to give evidence of biologic recovery.

That the XP12BE cells fail to excise a significant number of residues initially bound to their DNA allows us to compare the 4 carcinogens for intrinsic cytotoxic potency, i.e., the killing activity *per number of residues remaining unexcised at the "critical time"* which determines whether the cells can form a colony. From table 1 the intrinsic cytotoxic potency of *N*-AcO-AAS and *N*-AcO-PhAA are undoubtedly similar and comparable to that of UV-induced pyrimidine dimers and residues of 2 benzo[*a*]pyrene derivatives. Our data suggest that the intrinsic cytotoxicity of *N*-AcO-2-FAA is approximately fourfold less than that of the other agents; *N*-AcO-BPAA and *N*-AcO-2-FAA may be about equal. This conclusion is based on the data in text-figure 4 that suggest that some of the *N*-AcO-BPAA residues initially bound to XP12BE cells are subsequently lost.

The data in text-figures 4 and 5 comparing the rate of excision of DNA-bound residues by NF cells confirmed that the initial rate of removal of *N*-AcO-PhAA residues is twice as fast as that of *N*-AcO-BPAA or *N*-AcO-2-FAA, so that at an initial binding level of 40–50/10^6 nucleotides, approximately 40% of the *N*-AcO-PhAA residues are excised during the first 18 hours in the confluent state, whereas only about 16–20% of the *N*-AcO-2-FAA or *N*-AcO-BPAA residues are lost in that same period. If this same differential in excision rate is maintained by NF treated in confluence with concentrations of these 3 agents adjusted to yield the initial binding levels shown in table 1 and then released to form colonies, the number of unexcised residues remaining at the critical time may, as suggested earlier (*6, 11*), approximate the number remaining unexcised in the XP12BE cells. This would explain why 46 *N*-AcO-PhAA residues/10^6 nucleotides give approximately the same survival (37%) in NF as 3 residues/10^6 in the XP cells and why 4 *N*-AcO-AAS residues, which are excised so slowly by the NF, are sufficient to reduce their survival to 37%.

In summary, these studies correlate the rate of recovery of normal human cells from the potentially cytotoxic lesions induced by reactive ester derivatives of 4 aromatic amide carcinogens with their rate of excision of DNA adducts and demonstrate the lack of such recovery and excision in XP cells from complementation group A. These data imply that carcinogen residues bound to DNA are responsible for cell death and that their removal by excision repair as measured by loss of DNA-bound residues and the induction of repair replication results in higher levels of cell survival. The overall biologic effect on this excision repair is to decrease the ability of the initial number of carcinogen binding products to cause cell death in the excision-proficient normal cells. Results of unpublished studies with 2 of these agents (*N*-AcO-2-FAA and *N*-AcO-AAS) suggest that a similar decrease in potentially mutagenic DNA damage can also be attributed to excision repair in these human cells.

REFERENCES

(*1*) Roberts JJ: The repair of DNA modified by cytotoxic, mutagenic, and carcinogenic chemicals. *In* Advances in Radiation—Biology 7 (Lett JT, Adler H, eds). New York: Academic Press, 1978, pp 211–436

(*2*) Maher VM, Dorney DJ, Mendrala AL, et al: DNA excision repair processes in human cells can eliminate the cytotoxic and mutagenic consequences of ultraviolet irradiation. Mutat Res 62:311–323, 1979

(*3*) Konze-Thomas B, Levinson JW, Maher VM, et al: Correlation among the rates of dimer excision, DNA repair replication, and recovery of human cells from potentially lethal damage induced by ultraviolet radiation. Biophys J 28:315–326, 1979

(*4*) Petinga RA, Andrews AD, Tarone RE, et al: Typical xeroderma pigmentosum complementation group A fibroblasts have detectable ultraviolet light-induced unscheduled DNA synthesis. Biochim Biophys Acta 479:400–410, 1977

(*5*) Robbins JH, Kraemer KH, Lutzner JA, et al: Xeroderma pigmentosum: An inherited disease with sun sensitivity, multiple cutaneous neoplasms, and abnormal DNA repair. Ann Intern Med 80:221–248, 1974

(*6*) Maher VM, Curren RD, Ouellette LM, et al: Role of DNA repair in the cytotoxic and mutagenic action of physical and chemical carcinogens. *In* In Vitro Metabolic Activation in Mutagenesis Testing (deSerres FJ, Fouts JR, Bend JR, et al, eds). Amsterdam: Elsevier/North Holland, 1976, pp 313–336

(*7*) Simons JW: Development of a liquid-holding technique for the study of DNA repair in diploid fibroblasts. Mutat Res 59:273–283, 1979

(*8*) Maher VM, Hazard RM, Beland FJ, et al: Excision of the deacetylated C-8-guanine DNA adduct by human fibroblasts correlates with decreased cytotoxicity and mutagenicity. Proc Am Assoc Cancer Res 21:71, 1980

(*9*) Heflich RH, Hazard RM, Lommel L, et al: A comparison of the DNA binding, cytotoxicity and repair synthesis induced in human fibroblasts by reactive

derivatives of aromatic amide carcinogens. Chem Biol Interact 29:43–56, 1980

(*10*) HEFLICH RH, O'CALLAGHAN TG, SCRIBNER JD, et al: Correlation between excision of DNA adducts and recovery of human fibroblasts from the potentially lethal and mutagenic effects of *N*-acetoxy-4-acetylaminostilbene. Chem Biol Interact. In press

(*11*) MAHER VM, MCCORMICK JJ, GROVER PL, et al: Effect of DNA repair on the cytotoxicity and mutagenicity of polycyclic hydrocarbon derivatives in xeroderma pigmentosum human fibroblasts. Mutat Res 43:117–138, 1977

(*12*) HEFLICH RH, DORNEY DJ, MAHER VM, et al: Reactive derivatives of benzo[*a*]pyrene and 7, 12-dimethylbenz[*a*]-anthracene cause S_1 nuclease-sensitive sites in DNA and "UV-like" repair. Biochem Biophys Res Commun 77:634–641, 1977

(*13*) YANG LL, MAHER VM, MCCORMICK JJ: Error-free excision of the cytotoxic and mutagenic N^2-guanosine DNA adduct formed in human fibroblasts by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene. Proc Natl Acad Sci USA 77:5933–5937, 1980

(*14*) MAHER VM, BIRCH N, OTTO JR, et al: Cytotoxicity of carcinogenic aromatic amides in normal and xeroderma pigmentosum fibroblasts with different DNA repair capabilities. J Natl Cancer Inst 54:1287–1294, 1975

(*15*) CLEAVER JE: Defective repair replication of DNA in xeroderma pigmentosum. Nature 218:652–656, 1968

(*16*) _____: DNA repair and radiation sensitivity in human (xeroderma pigmentosum) cells. Int J Radiat Biol 18:557–565, 1970

(*17*) PAINTER RB, CLEAVER JE: Repair replication, unscheduled DNA synthesis, and the repair of mammalian DNA. Radiat Res 37:451–566, 1969

Discussion V [1]

L. Grossman: This session is now open for comments and questions. I have a question for Dr. Daune. Can you determine whether distribution of damages are random or nonrandom along the molecule?

M. Daune: So far, we have not resolved that problem, but we are studying it, of course.

Grossman: The paper by Dr. Poirier is open for discussion.

R. Ramanathan: You were talking of some toxicity because of your use of a direct-acting carcinogen, *N*-OH-2-FAA. Does it matter?

M. Poirier: No, it does not matter. It only matters in relation to the paraoxon. We believed it was important to establish that the paraoxon, combined with the carcinogen, was not wiping out the cells, and perhaps it was responsible for the effects that we observed.

Ramanathan: Does the paraoxon effect indicate that the deacetylation takes place only after binding from the DNA molecule?

M. Poirier: No; on the contrary, the paraoxon seems to be a specific inhibitor for a deacetylase enzyme that is associated with a nuclear membrane. This would indicate that the deacetylation actually takes place before the carcinogen is bound to DNA, i.e., as it is passing through.

Ramanathan: Are 3% of the cells acetylated?

M. Poirier: Three percent of the adducts that we find are acetylated.

Ramanathan: Acetylated?

M. Poirier: Yes, and 97% are deacetylated.

Ramanathan: If you stop the deacetylation by paraoxon, then you do not get any binding at all.

M. Poirier: Sometimes we get 1% of the control binding. We find that 99% or more of the binding is actually inhibited, which would argue that the deacetylation is necessary for the binding to DNA.

Grossman: The paper of Dr. Maher is now open for discussion.

R. Cameron: As a pathologist, I would like to raise a general question. I think it is an important one, which was raised by the elegant work in the talk, which considers not fibroblasts in vitro but the epidermal cells in the skin of the human animal exposed to sunlight and UV damage.

The question is: Which of the steps that we have learned about is the critical or the limiting factor when comparing the effects of UV exposure in the initiation and perhaps promotion of the development of skin cancers in the human animal? Again, the xeroderma pigmentosum model, the susceptible individual, is good for comparing this. If we consider the steps, first of all, activation, UV at least does not seem to need activation, excision repair, which you mentioned. Again, we see differences in these people. For the next step (cell proliferation), the skin, unlike the liver, is proliferating at all times, so that it would appear that some fixation must be going on because the rate of repair would be slower.

Then the question involves postreplication repair. Can we talk about this with reference to exposure to UV irradiation or carcinogens? This is the general area of questioning I am raising, with the human in mind.

Grossman: Excuse me, I think that is an excellent question for a discussion by the people who are participating in the repair research. It is most appropriate; I think that they all may want to participate.

Cameron: Perhaps we could save it until later.

Grossman: Yes, I think it might be more appropriate later, so we can move on.

E. C. Miller: If I understood right, Dr. Maher, you said that you had essentially only one adduct when you have the *N*-AcO-2-FAA. If that is so, what is the adduct?

V. Maher: We will give credit where credit is due. We have been working with Dr. Beland, investigating the adduct that we see. We see one adduct right now, and it appears to be the deacetylated C-8 adduct.

M. J. Griffin: I guess I am asking for another experiment. I am wondering if one could subculture the cells that already have a small amount of adduct fixed in them to find out if 1) they get rid of all of it, 2) their lifetimes are shortened, or 3) their ability to "transfer spontaneously" is enhanced. In other words, if you carry them like diploid fibroblasts, do they have a shorter time in culture? Have you done something to their total lifetimes, or do you get rid of all the carcinogen?

J. J. McCormick: We have not done such lifetime studies, and the problem with the radioactive carcinogen bound to the DNA is that you have so little of it left that you are unable to follow it in subsequent generations. Because you have more and more DNA, it increases the background problem. After a few generations, we are not able to follow it. It certainly would be interesting to see whether the lifetime is so much shorter in the cells.

Maher: Knowing that the XP-12 does not take any *N*-AcO-2-FAA adducts off, and, knowing those numbers when we get a 37% survival, we have 7 bound 10^6 nucleotides when the cells are released. They never take it off; it is still at that point when they go through the S-phase and into the M-phase. At that point, they are going to have one-half as much on, and then they are going to have a quarter, and so on. They will form a little clone, and soon you are going to dilute it. By the time you pick that clone,

Abbreviations: *N*-OH-2-FAA = *N*-hydroxy-2-fluorenylacetamide; *N*-AcO-2-FAA = *N*-acetoxy-2-FAA.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

you have 5,000 cells in that clone; it is still there somewhere in the midst.

Cameron: Perhaps I should rephrase the original question. I was trying to deal with the whole area of the development of skin cancer and, comparing the patients with xeroderma pigmentosum with the normal exposed population, get at the critical factor in this situation. It would seem that after exposure to the UV and because the skin cells or the epidermal cells are dividing constantly, what is critical to the difference would be the postreplication repair. I am wondering, in your system, Dr. McCormick, whether you see differences in the xeroderma pigmentosum cells versus the normal.

McCormick: Let me define postreplication repair again. It is not a repair process per se, it means the process by which a cell replicates DNA-containing lesions. How does it replicate DNA when lesions are in the way? That is the question one is asking. If it cannot replicate the DNA and it is lethal to the cell, postreplication repair in that sense is not taking place. You need a surviving cell at least to get cancer.

I do not think we can really answer the question of how this process takes place in cells. Sometime ago, I did a survey of the literature and found that, for as many people who had theories of how postreplication repair took place with UV irradiation, when they did studies with chemicals, the chemicals followed their mechanism. That made me suspicious that the theories were not as good as people had thought.

I really do not know how to answer that question. I think Dr. Liebermann may have something.

M. Liebermann: I have two thoughts. First I think one of the interesting things about skin is that, although skin cells are turning over and after you have labeled them with thymidine, you do not find all the basal cells labeled over any one period, e.g., 24 hours. In fact, there is turnover, but it is not like, let us say, the intestinal crypts. Therefore, a fair number of cells, by Dr. McCormick's and Dr. Maher's criteria, might be resting or at G_1 for an appreciable amount of time.

I think the other answer is that both postreplication and excision repair can be or probably are involved. I say that because many of the mutants, many of the people with xeroderma pigmentosum, are deficient in either repair system, but the phenotype, the proclivity for cancer, is not appreciably different in the two types. This finding supports the idea that both mechanisms may be involved.

Cameron: I think, in the future, what might be helpful when we study humans and experimental animals will be to develop in the skin a means of pulling out the initiated cells and determining their type. Are they the type of cells that Dr. Liebermann is talking about, which could, in fact, point to excision repair, i.e., xeroderma pigmentosum versus normal, as being critical to the initiation? In the future, I think this will be the key to the ability to develop models in the skin, like we have in the liver.

Ramanathan: Do you have a big difference in the cell cycling time between the xeroderma pigmentosum variants and the control cells?

McCormick: The cell cycling time varies by a couple of hours in the variants we used; there is not much difference at all between them. You can adjust the conditions so your normal cells grow a little more slowly, if you are concerned about that, so that both have the same cycling time.

W. Stott: I was wondering about the effect of so-called spontaneous depurination in this whole process of your cells. Do you see it? Is there a difference between the adducts?

McCormick: Presumably, spontaneous depurination is going on in all cells at all times. You will not see any difference in xeroderma pigmentosum or normal cells. If it is misrepaired and leads to mutations, you will have your background level of mutations; you always run controls in these experiments and subtract the background level of mutations for your results.

Stott: I was wondering if, at low concentrations of a compound such as 2-FAA with your xeroderma pigmentosum cells, there is a significant effect on the survival by the spontaneous depurination?

Maher: If by depurination, you mean the loss of the purine that happens to have the adduct on it, we have that data and we showed it. There is no loss of 2-FAA adducts from the xeroderma over a period of 6 days. Nothing changes and we have a fairly high specific activity. The survival does not change.

Grossman: I think with some of the alkylating agents, specifically at the N-7 position, which labilizes the N-glycosylic bond, you would expect, under those circumstances to see more mutations, but you do not.

Unidentified participant: Is there any reasonable way to bridge the gap between mutagenesis and carcinogenesis?

McCormick: I think you need to come back later and hear the papers on transformation experiments. You know that transformation experiments generally have the same protocols as mutagenesis experiments, but the end point is different. Generally, the way they differ is that many of them are highly selected systems and, other than the Syrian hamster embryo system, do not use normal cells directly obtained from an animal or a kind of primary cells. That is probably the best analogy, I would say.

A. Gregory: Dr. Maher, when you have the cells in confluence, is there any evidence for something else besides repair taking place during that confluence that may be responsible for the difference in survival after you plate the cells again?

Maher: Much may be happening that we do not know about, e.g., death and decay of RNA and proteins. The thing I would be most worried about would be cell turnover. If the surviving cells turn over, then when you examine them and ask how many are alive, you will note that there are twice as many as before. It would go from 20% to about 40%. We did all the studies to show that turnover was 0.5% or more during a period of 2 or 3 days. Besides that, we have an effective control, which is the xeroderma pigmentosum cell. If the control crept up toward 100% survival, we would be worried about whether it was repair or turnover, but it did not.

Grossman: Unless there are some provocative questions, I think we ought to go on to the discussion of the other

papers dealing with conformational changes. I shall take the prerogative of the chair and start the questions.

Some sensitive assays for the detection of the presence of various modified bases have been reported. We have seen some electron micrographs and some radioimmunoassays. One question that I think is of great interest, particularly to those of us who are working in repair, has to do with whether, in your DNA studies, you compare the DNA that has histone still bound to it, i.e., where the chromatin is providing a site that is protective either against your damage or against your antibodies. When you remove the protein, are those sites equally damaged as other DNA? I throw that open to those of you who have been studying conformation to see what suggestions you have.

R. Fuchs: We presently use oligonucleosomes of defined length and modify them in vitro with *N*-AcO-2-FAA; we remove the histones or proteins and then try to do this antibody-binding study to see whether any discrete type of distribution of the antibodies is occurring along the DNA molecules. Whether or not there is more fixation, I do not have the data.

Grossman: It is something you are thinking about.

Fuchs: Yes.

Grossman: I suppose the same approach could be used for following the distribution of repair. Once you know what the distribution is and if there is damage that is located and protected by a histone, it would be good to know if the damage is also protected against repair, at least in a steady state in which you can look at specific enzymes, or an S_1 sensitivity.

J. Scribner: Dr. Fuchs, you and Dr. Grunberger reported (summer 1979) on the relative mutagenicities of *N*-AcO-2-FAA and the iodo and fluoro forms in TA98. Did you investigate the complete spectra of the usual Ames' strains and note any shifts in the spectrum of sensitivity from the parent to the iodo compound?

Grunberger: We studied 2 strains, the TA98 and TA100.

Grossman: That was a short answer to a long question. I would like to make some suggestions for experiments for people. I think that the binding protein you have is good; it has no catalytic activity, but it has specificity.

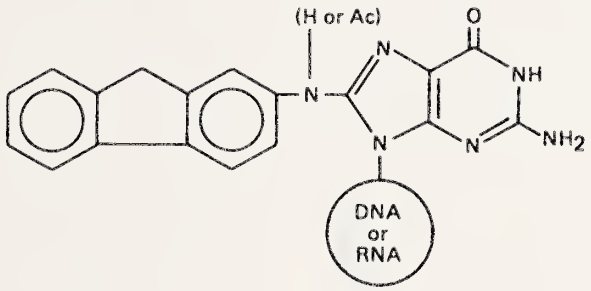
One interesting fact is that most cells seem to have these single-stranded specific endonucleases, and you wonder why they are in there. It is possible that the conformational distortions are significant, based upon what we have heard in the presentations and discussions.

The questions we all would like to ask are: Do these so-called nonspecific enzymes become specific if we were to get the single-stranded endonuclease from the mammalian cell with which we were working? Is it the function of these binding proteins to impose specificity? When we cause distortion, we not only distort one strand but are virtually causing a secondary distortion on the complementary strand. If you have a nonspecific single-stranded endonuclease, which strand does it work on? If it is going to be involved in excision, it obviously has to work on the strand that is damaged, not on the opposite undamaged one. Perhaps some of these binding proteins with no catalytic function may have a directive function on some of these nonspecific enzymes.

Another interesting study for those who have antibodies to a variety of adducts would be for them to determine whether the opposite strand can be protected. What is going on under these in situ conditions? You should be able to get some antibodies to help you see what is happening on the DNA. Then you could separate the chromatin and find out if those regions are protected, if the antibody can still bind, and so forth in vitro.

Are there any further questions? I have enjoyed this. I would like to thank the participants for an excellent series of discussions and papers.

Session VI: Mutagenesis and Transformation



Chairman: David Brusick

Mutagenicity of N-Substituted Aryl Compounds in Microbial Systems[1]

Snorri S. Thorgeirsson, Herman A. J. Schut, Norma Staiano, Peter J. Wirth, *and* Richard B. Everson[2]

ABSTRACT—Because many microbial test systems are available that scientists can use to detect genetic damage caused by chemicals or by their breakdown products (metabolites), they have been used to screen for potential chemical carcinogens in our environment, and to study the relationship between mutagenic and carcinogenic effects of chemicals at the mechanistic level. We have studied the mechanism by which N-substituted aryl compounds caused mutations in the *Salmonella* test system with *N*-hydroxy-2-fluorenylacetamide (*N*-OH-2-FAA) and *N*-hydroxyphenacetin (*N*-OH-Phen) as model compounds. The mutagenic activation of *N*-OH-2-FAA and *N*-OH-Phen by subcellular liver fractions from man, rat, mouse, and hamster proceeded exclusively by deacetylation and was catalyzed by either the microsomal, membrane-bound deacetylase (amidase) or the cytosolic *N*-*O*-acyltransferase. The *N*-hydroxy-2-fluorenamine, 2-nitrosofluorene, and *p*-nitrosophenetole were directly mutagenic in the *Salmonella* system; however, *N*-hydroxyacetaminophen was neither directly mutagenic nor mutagenic in the presence of subcellular liver fractions from laboratory animals and man. We attributed the lack of *N*-hydroxyacetaminophen mutagenicity to the instability of its proposed reactive form, *N*-acetyl-*p*-benzoquinone imine, under the conditions of the mutagenesis test. Therefore, we proposed that the mutagenic activation of aryl hydroxamic acids in the *Salmonella* system proceeded by deacylation and that the formation of the nitrenium ion within the bacteria from the aryl hydroxylamines was the final step in the mutagenic activation, although the formation of a free radical from these compounds cannot be excluded. Data are also presented that indicated both the frameshift and the base-pair mutation caused by derivatives of *N*-OH-2-FAA and *N*-OH-Phen were due to interaction with the same DNA bases.—Natl Cancer Inst Monogr 58: 229–236, 1981.

It is not surprising that increasing emphasis has recently been put on the study of the mutagenic potential of known and suspected chemical carcinogens because the most widely accepted theory on the etiology of cancer, i.e., the somatic mutation theory, stipulates that most if not all types of cancer originate from a gene mutation. These studies have for the most part been focused on two objectives: 1) the establishment of a rapid and sensitive screening method(s) for the detection of potential chemical carcinogens in our environment, and 2) a study of the relationship between mutagenic and carcinogenic effects of chemicals at the mechanistic level. For rather obvious reasons, the microbial mutagenesis test systems have been the most widely used research tools in the pursuit of these objectives, although increasing use of mammalian cell systems for mutagenesis testing is currently being advocated (*1*). We shall not deal with the screening aspects of chemical mutagenesis but rather concentrate on the use of microbial mutagenesis systems in studies in which we attempt to elucidate the mechanism by which chemicals, such as aromatic amines and amides, cause mutations and, to a lesser extent, we will discuss the relationship between the in vitro mutagenicity and the in vivo carcinogenic potential of these compounds.

MICROBIAL MUTAGENESIS TEST SYSTEMS

Many microbial test systems are available that researchers can use to detect genetic damage caused by chemicals or by their breakdown products or metabolites; some of these systems are listed in table 1. These test systems have the capacity to detect various mutational types (base-pair substitution, frameshift, etc.) and to mutate in both the backward (the mutant strain reverted back to its parent phenotype) and forward (the wild-type strain mutated to some measurable conditions) directions. However, none of these organisms contains the oxidative enzyme systems required for the metabolic activation of promutagens and/or procarcinogens. This led to the development by Ames and his co-workers (*5*) of the *Salmonella*/mammalian microsome test system in which subcellular fractions from rat liver provide the oxidative enzymes necessary for metabolic activation of promutagens and/or carcinogens. Inasmuch as the *Salmonella* test system is perhaps the most widely used of the microbial mutagenesis systems currently available for the evaluation of the mutagenic potential of chemicals and is also the system in which all our studies on the mutagenicity of aromatic amines and amides have been performed, we shall describe only this particular assay system in any detail.

In the *Salmonella* test system, also known as the Ames' system (*5*), four tester strains of *S. typhimurium* mutants that require histidine are commonly assayed for reversion to prototrophy after exposure to a potential mutagen (text-fig. 1). Each strain carries a different mutation in the

Abbreviations: HGPRT = hypoxanthine-guanine phosphoribosyltransferase; 2-FAA = *N*-2-fluorenylacetamide; OH = hydroxy; *N*-OH-2-FA = *N*-hydroxy-2-fluorenamine; *N*-OH-Phen = N-hydroxyphenacetin; *N*-OH-AP = *N*-hydroxyacetaminophen. aminophen.

[1] Presented at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7–9, 1979.

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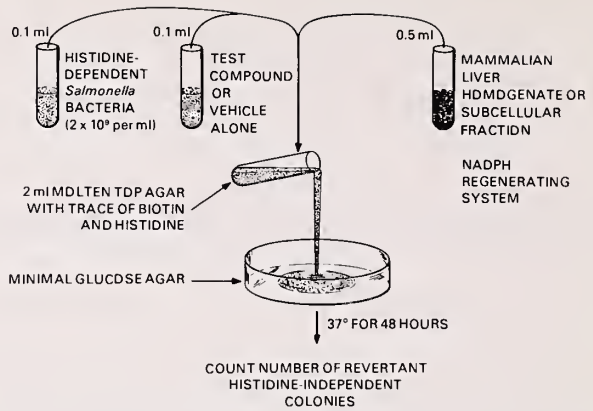
TABLE 1.—*Microbial mutagenesis test system*

Test system	References
Escherichia coli	(*2*)
E. coli (T_4 bacteriophage)	(*3*)
E. coli (prophage induction)	(*4*)
Salmonella typhimurium	(*5, 6*)
S. typhimurium (host-mediated)	(*7*)
Bacillus subtilis (transforming DNA)	(*8*)
B. subtilis (inhibition)	(*9*)
B. subtilis (spores)	(*10*)
Klebsiella pneumoniae	(*11*)
Citrobacter freundii	(*11*)
Dictyostelium discoideum	(*12*)
Streptomyces coelicolor	(*13*)
Saccharomyces cerevisiae	(*14*)
S. pombe	(*15*)
Aspergillus nidulans	(*16*)
Neurospora crassa	(*17*)



TEXT-FIGURE 1.—Schematic representation of the *Salmonella* system.

histidine operon, and each lesion has different molecular characteristics and thereby provides a wider target spectrum for interaction with potential mutagens. In addition, the *Salmonella* tester strains carry mutations that cause deletion of excision DNA repair and increase permeability of the cell membrane to organic molecules by elimination of the polysaccharide membrane barriers (*5*). Also, a plasmid has been incorporated into two strains (TA98 and TA100) to increase their sensitivity further (*18*).

As mentioned earlier, many chemicals become biologically active only after biotransformation by various drug-metabolizing enzymes. A metabolizing system composed of either the postmitochondrial supernatant (S-9 fraction) or the microsomal fraction from rat liver is most often included in the test system. However, the use of whole cells as the source of the metabolizing enzymes (metabolic activation and detoxification) has recently been declared feasible by Dybing et al. (*19*) and may reflect much better the actual metabolic processing of chemicals that occurs in vivo rather than that realized when subcellular fractions are used.

Many chemicals have been tested for mutagenicity in the *Salmonella* system, and McCann et al. (*20*) obtained positive results with a wide variety of compounds including aromatic amines, alkyl halides, polycyclic aromatic hydrocarbons, esters, epoxides, carbamates, nitrogen-containing aromatics and heterocyclic nitrosamines, fungal toxin, antibiotics, and azo and diazo compounds. Data obtained from scientists at other laboratories have since confirmed that diverse classes of compounds are mutagenic in this system. These include numerous drugs (*21*), food additives (*22*), and even extracts from fried meat and fish (*23, 24*).

Because the *Salmonella* tester strains are mutants in which the lesions are only partly known (i.e., the particular, single-base substitution is not known in TA1535 and TA100 but is known for the frameshift mutation in TA1537, TA1538, TA97 and TA98; *see* table 2), the question of mutagen specificity becomes important. A base-pair substitution mutation is the replacement of one nucleotide pair with another, and 6 interconversions are possible: two base-pair transitions (AT → GC and GC → AT) and four transversions (GC → CG; GC → TA; AT → CG; and AT → TA). Any one of these substitutions results in a change in one base in the mRNA codon, and this alteration may cause a change of one amino acid in the protein for which the gene codes. A frameshift mutation is caused by any lesion that shifts the reading frame of a gene either by inserting or deleting nucleotide pairs (as long as more or less than three pairs are inserted or deleted), which results in the synthesis of a different protein from the point of mutation onward. In this context, it is important to realize that a particular mutagen does not necessarily cause all kinds of mutation (this would also include mutations by large deletions). For example, mutagens specific for base-pair substitution will not revert frameshifted strains (*25, 26*). Moreover, there is evidence of specificity within the mutagenic classes; hydroxylamine causes only GC → AT transition, and 2-aminopurine causes AT → GC and GC → AT transitions but no transversions (*27*). It is also evident that structural specificity affects frameshift mutations, i.e., a mutagen may be active in mutating one DNA sequence but not another. This is substantiated by the strong mutagenic effect of 9-aminoacridine in the TA1537 strain compared with its lack of mutagenicity for the TA1538 (table 2).

At present, not all of the mutant strains are available to ensure that all possible base-pair interconversions and frameshifts can be detected. Several investigators, including ourselves, have attempted to solve the problem of mutagen–mutation specificity by using a forward mutation assay with the *Salmonella* tester strains [(*28–30*); Everson RB, Staiano N, Thorgeirsson SS: Manuscript in preparation]. The rationale for the use of the forward mutation assay is that, with a gene for an entire functional protein, the spectrum and sometimes the sensitivity to either base-pair or frameshift mutagens would be greatly increased. Our approach has been to use the *Salmonella* tester strains in the classical pour-plate system and to compare the specific backward mutation with a forward mutation by selecting for 8-azaguanine resistance [(*29*); Everson RB, Staiano N, Thorgeirsson SS: Manuscript in preparation], i.e., obtaining both *his*$^+$ and HGPRT-deficient mutants (table 2, text-fig. 2). In so doing, we can obtain quantita-

TABLE 2.—*Forward/backward mutation assay*

Backward

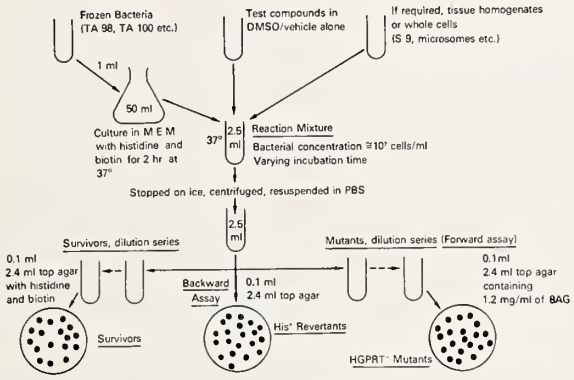
1) TA1535 and TA100
Mutation of unknown, single-base substitution

2) TA1537 and TA97
Mutation by loss of $\begin{matrix} -G-G- \\ -C-C- \end{matrix}$ sequence

3) TA1538 and TA98
Mutation by loss of $\begin{matrix} -G-C- \\ -C-G- \end{matrix}$ in $\begin{matrix} -G-C-G-C- \\ -C-G-C-G- \end{matrix}$ sequence

Forward

TA1535, TA1537, TA1538,
TA100, TA97, and TA98
Mutation by any base substitution, addition, or deletion that affects the function of HGPRT



TEXT-FIGURE 2.—Schematic representation of the forward/backward mutation assay.

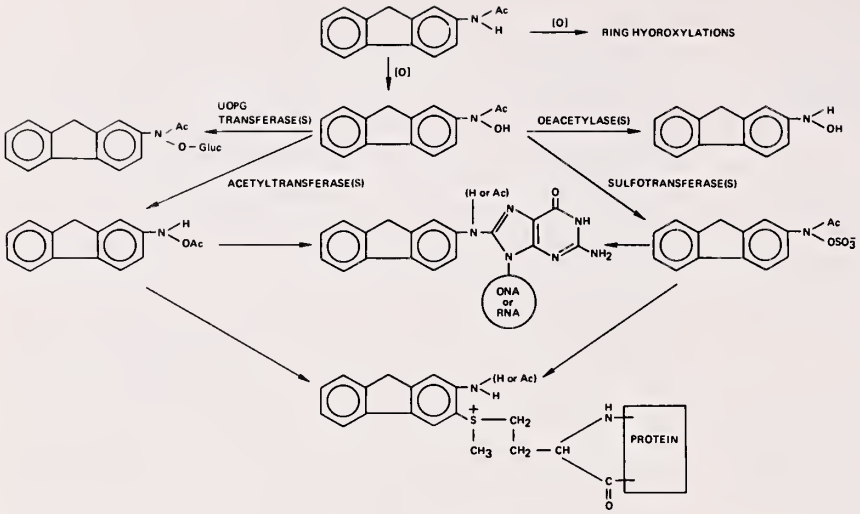
tive estimates of both types of mutations, which allows comparison with respect to sensitivity as well as the mechanism of mutation by the tested compounds.

MECHANISM OF IN VITRO MUTAGENICITY OF *N*-ACETYLARYLAMINES

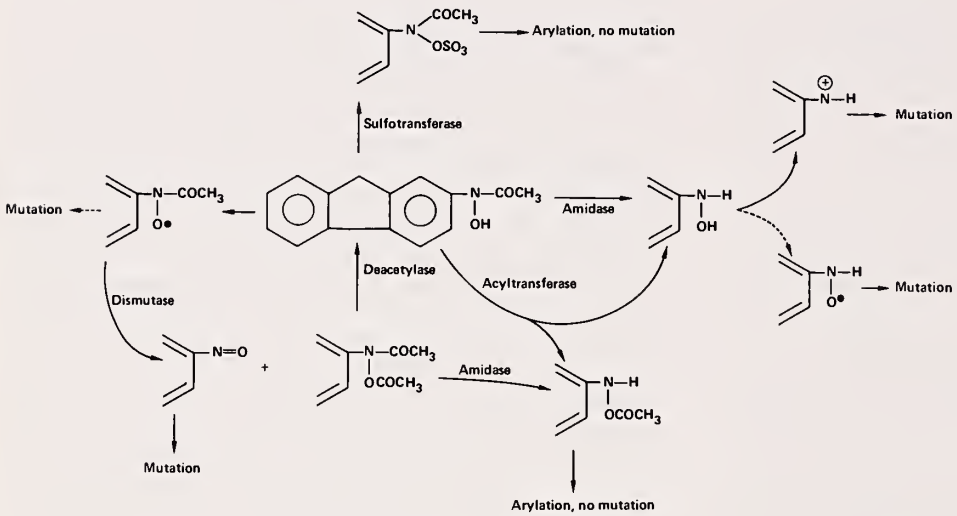
It is now recognized that the hepatoxicity and carcinogenicity of various *N*-acetylarylamines, such as 2-FAA, acetaminophen, and phenacetin, are exerted only after metabolic activation of these compounds to chemically reactive intermediates. The first step in this process occurs by a microsomal cytochrome P_{450}-dependent N-hydroxylation (*31–34*). Studies with subcellular fractions from different mouse strains (*35*) and guinea pigs (*36*) and inhibitors of cytochrome P_{450} (*37, 38*) have shown that the first and rate-limiting step (*39, 40*) in the mutagenic activation of 2-FAA is also mediated by a microsomal or a nuclear membrane-associated (*41*) cytochrome P_{450}-dependent N-hydroxylation. However, further metabolism of *N*-OH-2-FAA (a weak mutagen) is required before either the carcinogenic or the mutagenic effect can be expressed [text-fig. 3; (*38, 40, 42*)].

The metabolic activation steps usually considered to generate the ultimate carcinogenic form(s) of *N*-OH-2-FAA are catalyzed by the cytosolic enzymes sulfotransferase and *N*-*O*-acyltransferase, although the possible role of deacetylase, UDP glucuronyltransferase, and one-electron oxidation (i.e., radial formation) in the metabolic activation of *N*-OH-2-FAA cannot be ruled out (*42–45*). However, the mutagenic activation of *N*-OH-2-FAA in the *Salmonella* test system proceeds almost exclusively via deacetylation and is catalyzed by either the microsomal membrane-bound deacetylase (amidase) or the cytosolic *N*-*O*-acyltransferase (*40, 45–49*). The sulfotransferase does not appear to affect the mutagenic activation of *N*-OH-2-FAA in the *Salmonella* system whether the cytosolic fraction (*40, 48, 50*) or the purified sulfotransferase (Wirth PJ, Thorgeirsson SS: Unpublished data) from rat liver are used in the assay. The sulfate ester of *N*-OH-2-FAA, however, is mutagenic in the *B. subtilis* assay [transforming DNA; (*8*)] in which the ester reacts directly with naked bacterial DNA (*51*). Therefore, the instability of the *N*-OH-2-FAA sulfate ester in aqueous medium most likely prevents it from reaching and interacting with the bacterial DNA in the *Salmonella* system. Moreover, studies on the metabolic activation of *N*-OH-2-FAA with purified cell nuclei from rat liver have demonstrated that sulfotransferase neither contributed to the mutagenic activation nor to the covalent binding of *N*-OH-2-FAA to intranuclear nucleic acids and proteins (*40*), which further emphasizes the instability of this sulfate ester. Text-figure 4 shows the proposed mechanism for the in vitro mutagenic activation of *N*-OH-2-FAA in the *Salmonella* test system (*40*). Arguments in support of this proposal have already been introduced (*40*). The points to be emphasized are that deacetylation of *N*-OH-2-FAA, whether catalyzed by the membrane-bound deacetylase or the cytosolic *N*-*O*-acyltransferase, is the most important step in the mutagenic activation and that the formation of nitrenium ion within the bacteria from *N*-OH-2-FA appears to be the final step in the mutagenic activation, although the formation of a free radical from *N*-OH-2-FA cannot be excluded (*40*).

To establish whether the same mechanism for the mutagenic activation of *N*-OH-2-FAA (text-fig. 4) would apply to other aryl hydroxamic acids as well, we determined the mutagenicity of *N*-OH-Phen and *N*-OH-AP in the *Salmonella* system (*52*). The latter compound is neither directly mutagenic nor mutagenic in the presence of subcellular liver fractions from laboratory animals and man. The most likely explanation for the apparent lack of *N*-OH-AP mutagenicity is that the *N*-acetyl-*p*-benzoquinone imine, the proposed "toxic metabolic product" of *N*-OH-AP, is too unstable to reach the bacterial DNA. Similarly, *N*-OH-Phen is not directly mutagenic, but is mutagenic upon activation by subcellular liver and kidney fractions from man, rat, mouse, and hamster (*52*). Furthermore, it appears that the mutagenic activation of *N*-OH-Phen proceeds via the same mechanism by which *N*-OH-2-FAA is activated to a mutagen in vitro, i.e., deacetylation. This is illustrated in text-figure 5 in which paraoxon (diethyl-*p*-nitrophenylphosphate), like *N*-OH-2-FAA (*40, 48*), completely blocks the mutagenicity of *N*-OH-Phen. However, unlike *N*-OH-2-FAA, which is efficiently activated to a mutagen by the cytosolic enzyme *N*-*O*-acyltransferase (*40, 49*), *N*-OH-Phen is not activated to any substantial degree



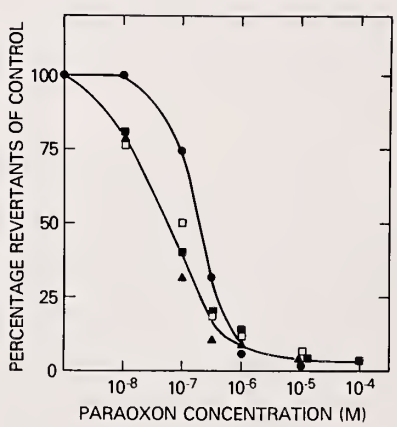
TEXT-FIGURE 3.—Metabolic pathways of 2-FAA.



TEXT-FIGURE 4.—Proposed mechanism of in vitro mutagenic activation of *N*-OH-2-FAA in the *Salmonella* test system.

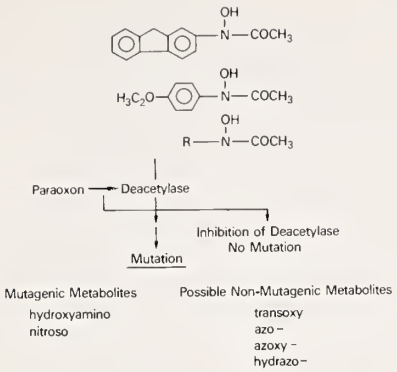
by rat or hamster liver 100,000 × *g* supernatant fractions (*52*). Therefore, we proposed that the mutagenic activation of aryl hydroxamic acids in the *Salmonella* system proceeds by deacylation (text-fig. 6).

Because a common mutagenic activation mechanism may apply to the aryl hydroxamic acids, we considered it worthwhile to determine whether the ultimate mutagenic species is the same and if the same type(s) of mutation is induced by these compounds. Sakai et al. (*40*) detected that the mutagenicity of *N*-OH-2-FAA as well as that of *N*-OH-FA and 2-nitrosofluorene is enhanced by the addition of ascorbic acid to the *Salmonella* system (text-fig. 7). In contrast, ascorbic acid inhibits, in a dose-response fashion, the mutagenicity of *N*-OH-Phen (*52*) and *p*-nitrosophenetole (text-fig. 7). A possible interpretation of these data would be that the ultimate mutagenic species derived from *N*-OH-Phen is different from that derived from *N*-OH-2-FAA (a free radical vs. a nitrenium ion). However, it seems equally possible for one to explain the inhibitory effect of ascorbic acid on the mutagenicity of *N*-OH-Phen and *p*-nitrosophenetole solely on the basis of chemical interactions. Ascorbic acid may simply be reducing either *N*-hydroxyphenetidine to intermediates



TEXT-FIGURE 5.—Effect of paraoxon on the mutagenic activation of *N*-OH-Phen in TA100 by C57BL/6N mouse liver S-9 fraction, hamster liver microsomes, human liver microsomes, and rat kidney S-9 fractions.

which react with *p*-nitrosophenetole to form the nonmutagenic compounds azoxyphenetole and azophenetole [text-fig. 8; (*52*)]. It is also possible that these chemical reactions



TEXT-FIGURE 6.—Proposed mutagenic activation of aryl hydroxamic acids in the *Salmonella* system.

(text-fig. 8) would proceed much more easily for *N*-OH-Phen than for *N*-OH-2-FAA due to the inductive effect of the ethoxy group of *N*-OH-Phen. These data could support the proposal that the ultimate mutagenic species derived from both *N*-OH-Phen and *N*-OH-2-FAA might have the same chemical characteristics.

Although *N*-OH-2-FAA and *N*-OH-Phen cause frameshift and base-pair substitution mutations in the *Salmonella* system, the relative frequency of these mutations for the 2 compounds is different because *N*-OH-2-FAA is two to three times more effective in causing frameshift than base-pair mutations, and *N*-OH-Phen is ten times more active in causing base-pair mutation than it is in inducing frameshift mutation (table 3).

The mutation of TA100 induced by *N*-OH-2-FAA and *N*-OH-Phen is caused by a reversion of an unknown, single-base substitution (table 2). Inasmuch as Corbett et al. (*53*) found that both *N*-acetoxy-2-FAA and 7-fluoro-*N*-acetoxy-2-FAA caused A-T → G-C base-pair transitions as well as frameshift mutations in the T_4 bacteriophage, it seems likely that the same base-pair transition could be brought about by *N*-OH-2-FAA and *N*-OH-Phen in the *Salmonella* system after covalent interaction with deoxyadenosine in the bacterial DNA. The possible importance of the interaction of these compounds with deoxyadenosine in their mutagenic effect is further indicated by the results obtained in the forward/backward assay (table 3) in which the mutagenicity ratio of *N*-OH-2-FAA and *N*-OH-Phen is the same in both directions.

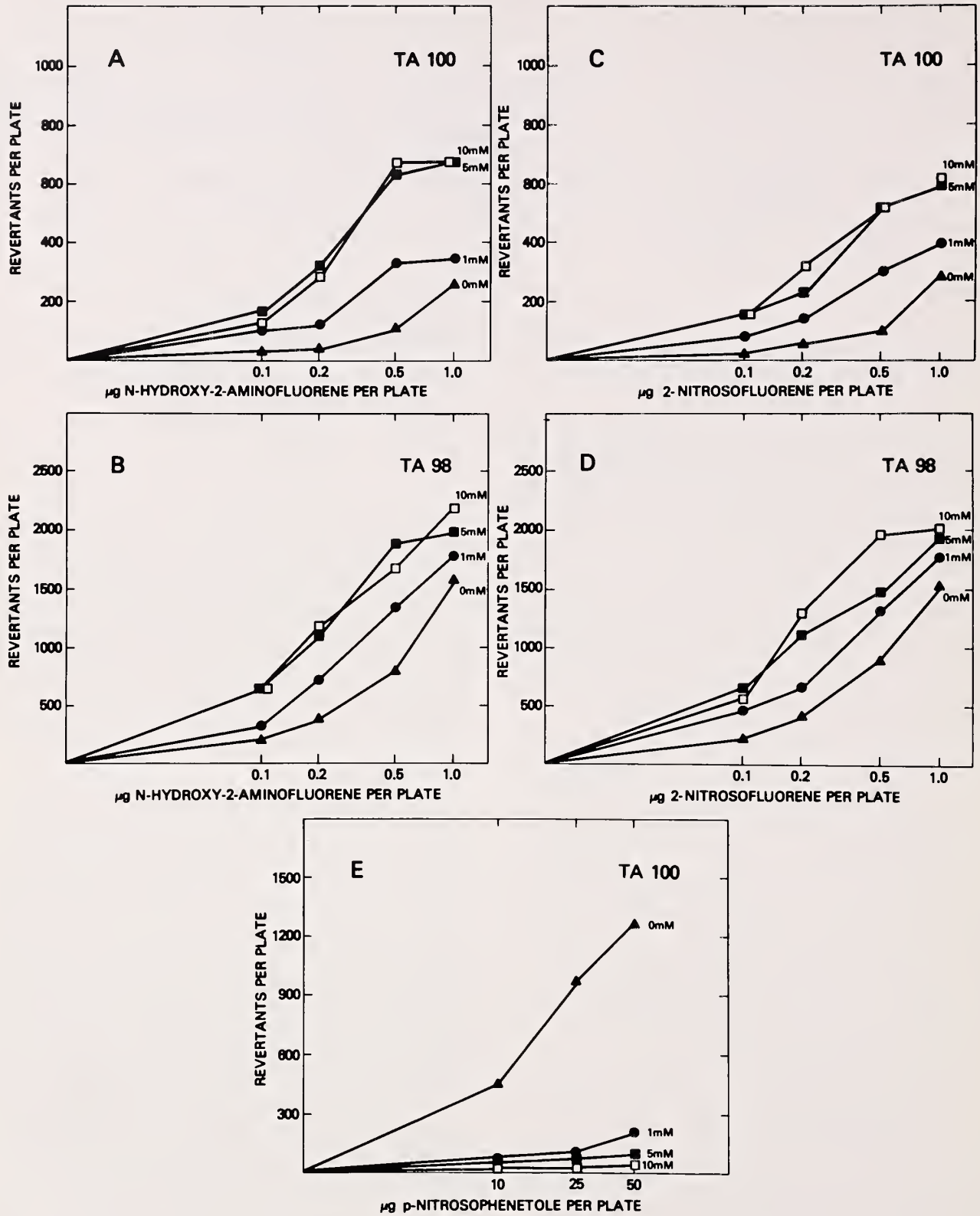
If a compound is to induce a frameshift mutation in TA1538 and/or TA98, it has to cause a loss of GC/CG in a G-C-G-C-sequence (table 2). Because *N*-OH-2-FAA covalently binds both in vivo and in vitro to the C-8 and N-2 position of guanine (*54, 55*), it seems reasonable to assume that the loss of GC/CG is caused by the covalent interaction of the *N*-OH-2-FAA-derived species with guanine. By far the largest part of the in vivo nucleic acid adduct of FAA and/or *N*-OH-2-FAA in the rat liver is deacetylated (*54, 56*). Similarly, one would expect that after being metabolically activated *N*-OH-Phen would cause the frameshift mutation in TA98 by covalently interacting with guanine. Further studies with *N*-OH-Phen are needed for clarification of the interactions of this compound with nucleic acids.

TABLE 3.—*Mutagenicity of* N-*OH-FAA and* N-*OH-phen in* Salmonella *test systems*

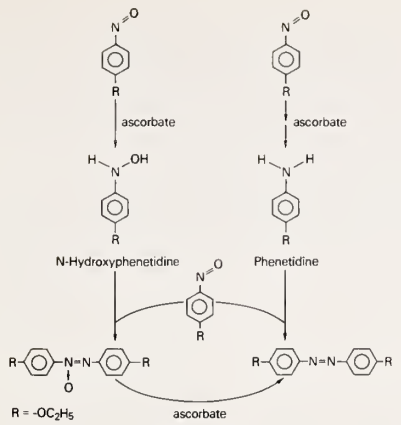
Test system	Bacterial strain	Compound	Revertant/nmol	Mutagenicity ratio
Ames	TA98	*N*-OH-2-FAA	120	6,667
Ames	TA98	*N*-OH-Phen	0.018	
Ames	TA100	*N*-OH-2-FAA	60	357
Ames	TA100	*N*-OH-Phen	0.168	
Forward/backward				
Backward	TA100	*N*-OH-2-FAA	47	390
Backward	TA100	*N*-OH-Phen	0.120	
Forward	TA100	*N*-OH-2-FAA	41	387
Forward	TA100	*N*-OH-Phen	0.106	

The relationship between the in vitro mutagenicity of a compound and its carcinogenic potential has been a matter of extensive debate (*57*). It is beyond the scope of this paper to deal with the relationship between in vitro mutagenicity versus in vivo carcinogenicity of aromatic amines and amides to any extent. However, we would like to conclude by showing how studies on the mechanism of in vitro mutagenicity of aromatic amides may in fact throw light on the mechanism by which these compounds induce carcinogenesis in vivo.

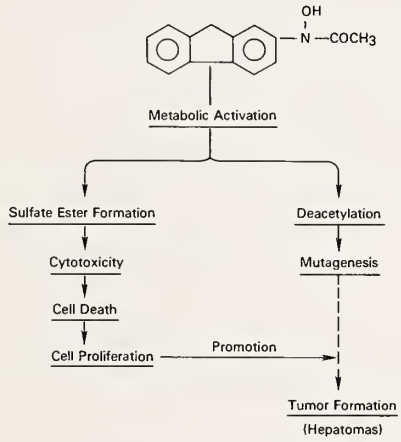
The incidence of hepatomas in rats and mice induced by FAA is strongly associated with the hepatic sulfotransferase activity, and the sulfate ester of *N*-OH-2-FAA has been proposed as one of the ultimate carcinogenic forms of FAA in the rat liver (*42, 43*). Our data on the mutagenicity and covalent binding of *N*-OH-2-FAA to nuclear nucleic acids of *N*-OH-2-FAA do, however, show that the sulfate ester of *N*-OH-2-FAA is not contributing to either the mutagenicity or the covalent binding to the nucleic acids of this compound (*41, 51*). Therefore, these results and the fact that 2-FAA and/or *N*-OH-2-FAA cause tumors in organs lacking sulfotransferase activity have provided a reason for our reexamining the role of the sulfotransferase in the induction of liver cancer by 2-FAA and/or *N*-OH-2-FAA. Inasmuch as the activity of liver sulfotransferase is an important factor in the determination of the hepatotoxicity of *N*-OH-2-FAA (*58*), we proposed that the role of the sulfotransferase in the hepatocarcinogenesis of *N*-OH-2-FAA is due primarily to the cytotoxic effects of the sulfate ester, which, in turn, leads to cell proliferation and thereby promotes the "initiated" cells [text-fig. 9; (*40*)]. The metabolic pathways that lead to initiation of liver tumors in the rat remain to be clearly established, although it is likely that *N*-O-acyltransferase and deactylase may, as is the case in the mutagenesis process, be important in the initiation of 2-FAA and *N*-OH-2-FAA carcinogenesis.



TEXT-FIGURE 7.—Effect of ascorbic acid on the mutagenicity of *N*-OH-2-FA, 2-nitrosofluorene, and *p*-nitrosophenetole.



TEXT-FIGURE 8.—Proposed mechanism for inhibition of *p*-nitrosophenetole-induced mutagenesis by ascorbic acid.



TEXT-FIGURE 9.—Possible role of sulfotransferase in the carcinogenesis by *N*-OH-2-FAA in rat liver.

REFERENCES

(*1*) WILLIAMS GM: Mammalian culture systems for the study of genetic effects of N-substituted aryl compounds. Natl Cancer Inst Monogr 58:237–242, 1981

(*2*) BRIDGES BA: Simple bacterial systems for detecting mutagenic agents. Lab Pract 21:413–416, 1972

(*3*) HARTMAN PE, LEVINE K, HARTMAN Z, et al: Hycanthone: A frameshift mutagen. Science 172:1058–1060, 1971

(*4*) GOLDSCHMIDT FP, MILLER R, MATNEY ST: Induction of prophage in a lysogen of a deep rough strain of *E. coli.* A possible method for detecting carcinogens. Microbiol Genet Bull 41:3–5, 1976

(*5*) AMES BN: The detection of chemical mutagens with enteric bacteria. *In* Chemical Mutagens: Principles and Methods for Their Detection (Hollaender A, ed), vol 1. New York: Plenum Press, 1971, pp 267–283

(*6*) AMES BN, MCCANN J, YAMASAKI E: Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenesis test. Mutat Res 31:347–351, 1975

(*7*) LEGATOR MS, MALLING HV: The host-mediated assay, a practical procedure for evaluating potential mutagenic agents in mammals. *In* Chemical Mutagens: Principles and Methods for Their Detection (Hollaender A, ed), vol 2. New York: Plenum Press, 1971, pp 569–591

(*8*) FREESE E, STRACK HB: Induction of mutations in transforming DNA by hydroxylamine. Proc Natl Acad Sci USA 48:1796–1803, 1962

(*9*) KADA T: *Rec* assay with cold incubation with and without metabolic reactivation in vitro [proceedings]. Mutat Res 38:340, 1976

(*10*) MACGREGOR JT, SACKS LE: The sporulation system of *Bacillus subtilis* as the basis of a multi-gene mutagen screening test. Mutat Res 38:271–286, 1976

(*11*) KRAMERS PG, KNAPP AG, VOOGD CE: Lack of mutagenicity of chlormequat chloride in *Drosophila* and in bacteria. Mutat Res 31:65–67, 1976

(*12*) LIVERANT IJ, PEREIRA DA, SILVA LH: Comparative mutagenic effects of ethyl methanesulfonate, *N*-methyl-*N'*-nitro-*N*-nitroso-guanine, ultraviolet reduction and caffeine on *Dictyostelium discoideum.* Mutat Res 33:135–146, 1976

(*13*) CARERE A, MORPURO G, CARDAMONE G, et al: Point mutation induced by pharmaceutical drugs. Mutat Res 29:237–241, 1975

(*14*) CHAMBERS C, DUTLA SK: Mutagenic tests of chlordane on different microbial tester strains. Genetics 83(Suppl): 13–24, 1976

(*15*) LOPRIENO N, BARALE R, BARONCELLI S, et al: The use of yeast genetic systems in environmental mutagenesis. Mutat Res 29:237, 1975

(*16*) KAFTER E, MARSHALL P, COHEN G: Well-marked strains of *Aspergillus* for tests of environmental mutagens: Identification of induced mitotic recombination and mutation. Mutat Res 38:141–156, 1976

(*17*) DE SERRES FJ, MALLING HV: Measurement of recessive lethal damage over the entire genome and at two specific loci in the ad-3 region of a two-component heterokaryon of *Neurospora crassa. In* Chemical Mutagens: Principles and Methods for Their Detection (Hollaender A, ed), vol 2. New York: Plenum Press, 1971, pp 311–343

(*18*) MCCANN J, SPINGARN NE, KOBORI J, et al: Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc Natl Acad Sci USA 72:979–983, 1975

(*19*) DYBING E, SODERLAND E, HAUG LT, et al: Metabolism and activation of 2-acetylaminofluorene in isolated rat hepatocytes. Cancer Res 39:3268–3275, 1979

(*20*) MCCANN J, CHOI E, YAMASAKI E, et al: Detection of carcinogens as mutagens in the *Salmonella*–microsome test: assay of 300 chemicals. Proc Natl Acad Sci USA 72:5135–5139, 1975

(*21*) DYBING E, SAXHOLM HJ, AUNE T, et al: Studies on mutagenic and carcinogenic N-substituted aryl compounds: Cosmetics and drugs. Natl Cancer Inst Monogr 58:21–26, 1981

(*22*) VENITT S, BUSHELL CT: Mutagenicity of the food colour brown FK and constituents in *Salmonella typhimurium.* Mutat Res 40:309–316, 1976

(*23*) NAGAO M, HONDA M, SERIO Y, et al: Mutagenicity of protein pyrolysates. Cancer Lett 2:335–339, 1977

(*24*) SUGIMURA T, NAGAO M: Carcinogenic, mutagenic, and comutagenic aromatic amines in human foods. Natl Cancer Inst Monogr 58:27–33, 1981

(*25*) OESCHGER NS, HARTMAN PE: ICR-induced frameshift mutations in the histidine operon of *Salmonella.* J Bacteriol 101:490–504, 1970

(*26*) WHITFIELD HJ JR, MARTIN RG, AMES BN: Classification of aminotransferase (*e* gene) mutants in the histidine operon. J Mol Biol 21:335–355, 1966

(*27*) FREESE E: Molecular mechanism of mutations. *In* Chemical Mutagens: Principles and Methods for Their Detection

(Hollaender A, ed), vol 1. New York: Plenum Press, 1971, pp 1–57

(*28*) Ames BN, Lee FD, Durston WE: An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci USA 70:782–786, 1973

(*29*) Skopek TR, Liber HL, Krolewski JJ, et al: Quantitative forward mutation assay in *Salmonella typhimurium* using 8-azaguanine resistance as a genetic marker. Proc Natl Acad Sci USA 75:410–414, 1978

(*30*) Skopek TR, Liber HL, Kaden DA, et al: Relative sensitivities of forward and reverse mutation assays in *Salmonella typhimurium*. Proc Natl Acad Sci USA 75:4465–4469, 1978

(*31*) Thorgeirsson SS, Jollow DJ, Sasame HA, et al: The role of cytochrome P_{450} in N-hydroxylation of 3-acetylaminofluorene. Mol Pharmacol 9:398–404, 1973

(*32*) Potter WZ, David DC, Mitchell JR, et al: Acetaminophen-induced hepatic necrosis. III. Cytochrome P_{450}-mediated covalent binding in vitro. J Pharmacol Exp Ther 187:203–210, 1973

(*33*) Hinson JA, Mitchell JR: N-hydroxylation of phenacetin by hamster liver microsomes. Drug Metab Disp 4:430–435, 1976

(*34*) Thorgeirsson SS, Felton JS, Nebert DW: Genetic differences in the aromatic hydrocarbon-inducible N-hydroxylation of 2-acetylaminofluorene and acetaminophen-produced hepatotoxicity in mice. Mol Pharmacol 11:159–165, 1975

(*35*) Thorgeirsson SS, Nebert DW: The *Ah* locus and the metabolism of chemical carcinogens and other foreign compounds. Adv Cancer Res 25:149–193, 1977

(*36*) Takeishi K, Okuno-Kaneda S, Seno T: Mutagenic activation of 2-acetylaminofluorene by guinea-pig liver homogenates: Essential involvement of cytochrome P_{450} mixed-function oxidases. Mutat Res 62:425–437, 1979

(*37*) Felton JS, Nebert DW: Mutagenesis of certain activated carcinogens in vitro associated with genetically mediated increases in monooxygenase activity and cytochrome P_{450}. J Biol Chem 250:6769–6778, 1975

(*38*) Felton JS, Nebert DW, Thorgeirsson SS: Genetic differences in 2-acetylaminofluorene mutagenicity in vitro associated with mouse hepatic aryl hydrocarbon hydroxylase activity induced by polycyclic aromatic compounds. Mol Pharmacol 12:225–233, 1976

(*39*) Nelson WL, Thorgeirsson SS: Structural requirements for mutagenic activity of 2-acylaminofluorenes in the *Salmonella* test system. Biochem Biophys Res Commun 71:1201–1206, 1976

(*40*) Sakai S, Reinhold CE, Wirth PJ, et al: Mechanism of in vitro mutagenic activation and covalent binding of *N*-hydroxy-2-acetylaminofluorene in isolated liver cell nuclei from rat and mouse. Cancer Res 38:2008–2067, 1978

(*41*) Gutmann HR, Malejka-Giganti D, Barry EJ, et al: On the correlation between the hepatocarcinogenicity of the carcinogen, *N*-2-fluorenylacetamide, and its metabolic activation by the rat. Cancer Res 32:1554–1561, 1972

(*42*) Miller JA: Carcinogenesis by chemicals: An overview. G.H.A. Clowes Memorial Lecture. Cancer Res 30:559–579, 1970

(*43*) DeBaun JR, Miller EC, Miller JA: *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis in protein-(methion-*S*-yl) binding in rat liver. Cancer Res 30:577–595, 1970

(*44*) King CM: Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. Cancer Res 34:1503–1515, 1974

(*45*) Kriek E: Carcinogenesis by aromatic amines. Biochim Biophys Acta 355:177–203, 1974

(*46*) Felton JS, Nebert DW, Thorgeirsson SS: Genetic differences in 2-acetylaminofluorene mutagenicity in vitro associated with mouse hepatic aryl hydrocarbon hydroxylase induced by polycyclic aromatic compounds. Mol Pharmacol 12:225–234, 1976

(*47*) Stout DL, Baptist JN, Mahoney TS, et al: *N*-Hydroxy-2-aminofluorene: The principal mutagen produced from *N*-hydroxy-2-acetylaminofluorene by a mammalian supernatant enzyme preparation. Cancer Lett 1:269–274, 1976

(*48*) Schut HA, Wirth PJ, Thorgeirsson SS: Mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system: The role of deacetylation by liver and kidney fraction from mouse and rat. Mol Pharmacol 14:682–692, 1978

(*49*) Weeks CE, Allaben WT, Louie SC, et al: Role of arylhydroxyamic acid acyltransferase in the mutagenicity of *N*-hydroxy-*N*-2-fluorenylacetamide in *Salmonella typhimurium*. Cancer Res 38:613–618, 1978

(*50*) Mulder GH, Hinson JA, Nelson WL, et al: The role of sulfotransferase from rat liver in the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in *Salmonella typhimurium*. Biochem Pharmacol 36:1356–1358, 1977

(*51*) Maher VM, Miller EC, Miller JA, et al: Mutation and decrease in density of transforming DNA produced by derivatives of the carcinogens 2-acetylaminofluorene and *N*-methyl(4-aminoazobenzene). Mol Pharmacol 4:441–456, 1968

(*52*) Wirth PJ, Dybing E, von Bahr C, et al: Mechanism of *N*-hydroxyacetylarylamine mutagenicity in the *Salmonella* test system: Metabolic activation of *N*-hydroxyphenacetin by liver and kidney fractions from the rat, mouse, hamster, and man. Mol Pharmacol 18:117–127, 1980

(*53*) Corbett TH, Heidelberger C, Dove WF: Determination of the mutagenic activity to bacteriophage T_4 of carcinogenic and noncarcinogenic compounds. Mol Pharmacol 6:667–679, 1970

(*54*) Kriek E, Westra JG: Formation of *N*-2-fluorenylhydroxylamine adducts of DNA in vivo and in vitro and some of their properties. Natl Cancer Inst Monogr 58:139–142, 1981

(*55*) Grunberger D: *N*-2-Fluorenylacetamide-induced conformational and functional damage to DNA. Natl Cancar Inst Monogr 58:193–199, 1981

(*56*) Poirier MC, Yuspa SH: Detection and quantitation of acetylated and deacetylated *N*-2-Fluorenylacetamide-DNA adducts by radioimmunoassay. Natl Cancer Inst Monogr 58:211–216, 1981

(*57*) Ames BN: Identifying environmental chemicals causing mutations and cancer. Science 204:587–593, 1979

(*58*) Irving CC: Comparative toxicity of *N*-hydroxy-2-acetylaminofluorene in several strains of rats. Cancer Res 35: 2959–2961, 1975

Mammalian Culture Systems for the Study of Genetic Effects of N-Substituted Aryl Compounds[1, 2]

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ABSTRACT—Liver-derived intact cell systems are useful for examination of the genetic effects of N-substituted aryl compounds. With freshly isolated hepatocytes, the hepatocyte primary culture–DNA repair test detects the genotoxicity of a spectrum of activation-dependent aryl amines and amides. Likewise, continuous lines of adult rat liver epithelial cells are mutated at the hypoxanthine-guanine phosphoribosyl locus by such compounds. Hepatocyte-mediated mutagenesis enhances the activation of aromatic amines to mutagenic metabolites. These intact cell systems provide a balance of detoxification and activation reactions for evaluation of tissue profiles of metabolism and capability for the production of genotoxic metabolites.—Natl Cancer Inst Monogr 58: 237–242, 1981.

CARCINOGEN METABOLISM IN CULTURE SYSTEMS

Subcellular fractions have been used extensively for studies of carcinogen metabolism (*1–8*) and the generation of mutagens (*9–12*). A great deal of important information on the mechanism of action of N-substituted aryl compounds has been obtained from such studies (*4, 13–22*). Nevertheless, enzyme preparations have an inherent deficiency for the study of overall carcinogen metabolism; unless specifically modified, they do not perform conjugation reactions and may be lacking in other detoxification pathways. Deficiencies in processes of carcinogen detoxification were suggested (*23*) to account for the fact that, whereas pretreatment of animals with microsomal enzyme inducers inhibits carcinogenesis by activation-dependent chemicals in vivo, such pretreatment paradoxically enhances the generation of mutagens by subcellular enzyme preparations. The detoxification pathways induced by pretreatment that result in decreased carcinogenicity were postulated to be lost selectively in microsomal preparations which would leave a net enhancement of activation. This suggestion was supported by a study (*24*) in which we documented that postmitochondrial supernatants from normal liver had little capacity to metabolize benzo[*a*]pyrene to water-soluble conjugates and that conjugation was only slightly increased in preparations from 3-methylcholanthrene-induced rats. In contrast, the intact hepatocytes from normal animals formed substantial amounts of conjugated products, and this formation was markedly increased after enzyme induction. The substantial level of detoxification reactions in intact hepatocytes, also reported by Dybing et al. (*25*) for 2-FAA, thus corresponds to the in vivo situation in which detoxification usually predominates over activation. Therefore, intact cell systems are essential for a realistic representation of the in vivo pattern of carcinogen metabolism and the determination of tissue capability for activation of chemical carcinogens.

Intact cell systems derived from the principal target organs in rodent animal models for carcinogenicity of N-substituted aryl compounds, i.e., liver, bladder, intestine, mammary gland, and ear duct, have not been extensively developed. Organ culture systems have been reported for liver, mammary gland, colon, and bladder, but, apart from the liver studies to be described, investigators have used only cultures of the mammary gland to examine the action of N-substituted aryl compounds (*26*). This is an area that will be of considerable interest for future studies.

Liver-derived cell systems, either suspensions of freshly isolated hepatocytes or primary cultures, have been used extensively in the study of metabolism (*25, 27, 28*) and activation of arylamines (*25, 29–38*). Primary cultures of hepatocytes are used in a screening test for chemical carcinogens that was developed in our laboratory (*32, 36, 39*). In this test, called the "HPC/DNA repair test," carcinogens are detected by their ability to elicit DNA repair measured autoradiographically as unscheduled DNA synthesis in nonreplicating cultures of hepatocytes. This test has demonstrated sensitivity with a number of N-substituted aryl compounds (table 1). Similar results have also been reported by other groups who also used hepatocytes (*33–35, 38*). The ability of the HPC/DNA repair test to function in the absence of inhibitors of replicative DNA synthesis, such as hydroxyurea, that are used in some approaches (*34*) presents a major advantage in the screening

Abbreviations: 2-FAA = *N*-2-fluorenylacetamide; HPC = hepatocyte primary culture(s); *N*-OH-2-FAA = *N*-hydroxy-2-FAA; 2-FA = 2-fluorenamine; *N*-AcO-2-FAA = *N*-acetoxy-2-FAA; ARL = adult rat liver; HGPRT = hypoxanthine-guanine phosphoribosyltransferase; AG = 8-azaguanine; TG = 6-thioguanine; CFC = colony-forming cells.

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TABLE 1.—*Results in the HPC/DNA repair test with N-substituted aryl compounds*

Compounds	Carcinogenicity[a]	HPC/DNA repair test[b]		
		Results	Grain/nucleus	Dose, M
2-FAA	+	+	42.4 ± 4.9	10^{-3}
4-FAA	?	+	12.3 ± 1.2	10^{-3}
2-FA	+	+	28.1 ± 3.6	10^{-4}
9-FA	−	−	0.5	10^{-4}
3-Methyl-2-naphthylamine	+	+	11.3	10^{-5}
Biphenyl-4,4′-diamine (benzidine)	+	+	27.8 ± 3.7	10^{-5}
2′,3-Dimethyl-4-aminobiphenyl	+	+	54.1 ± 19.3	10^{-5}
4-Aminobiphenyl	+	+	71.8 ± 36.1	10^{-6}
2-Aminobiphenyl	−	−	2.0 ± 1.9	10^{-5}
4,4′-Methylene-bis-2-chloroaniline	+	+	32.6 ± 1.7	4×10^{-4}
Aniline	−	−	0.1 ± 0	10^{-3}

[a] Plus sign = carcinogenic; minus sign = noncarcinogenic.

[b] All results represent the highest of those previously reported or unpublished; mean ± SD of triplicate cover slips.

of N-substituted aryl compounds. Brandt et al. (*40*) have shown that these aryl compounds interfere with the blocking of DNA synthesis by hydroxyurea and thereby permit its resumption, which may be confused with repair synthesis; because the HPC/DNA repair test does not require hydroxyurea this complication is avoided.

In other studies, replicating liver epithelial cell lines have undergone malignant transformation in response to *N*-OH-2-FAA (*29*) and displayed DNA fragmentation (*30*) and chromosome aberrations (*37*) after exposure to 2-FAA.

MAMMALIAN CELL MUTAGENESIS STUDIES

The use of cultured mammalian cells for investigation of the mutagenicity of N-substituted aryl compounds has been limited (table 2). Moreover, none of the cell lines studied (*41–45*) has demonstrated a significant capability to activate aromatic amines. Although this limitation can be overcome by the addition of subcellular enzyme preparations (*44*) as in the bacterial mutagenesis assays, the use of intact cell systems would be more desirable for a balanced profile of metabolism. Therefore, we examined the ability of liver cell systems to generate mutagenic metabolites from 2-FAA.

The mutagenesis assay developed in our laboratory involves continuous lines of ARL epithelial cells that are derived from primary cultures of hepatocytes obtained by perfusion and dissociation of ARL (*46*). The lines are clearly epithelial because they form adenocarcinomas upon malignant conversion (*47*). They possess some of the same properties as hepatocytes but at reduced levels (*31*). Importantly, like hepatocytes, they have a low level of γ-glutamyltranspeptidase, which is increased upon transformation (*48*). The assay developed in these lines (*49–51*) tests for mutations at the HGPRT locus and is referred to as the "ARL/HGPRT mutagenesis assay." The HGPRT is required for the utilization of exogenous purines. Mutants deficient in HGPRT are therefore resistant to the toxic effects of synthetic purine analogs and can be selected by exposure of cultures to toxic analogs such as AG and TG. The conditions devised for the use of this assay (*51*) include 1) 3-day exposure to activation-dependent carcinogens, 2) a minimum of 14 days for induced mutant expression, 3) seeding density of 1×10^4 cells/cm² and use of TG for the selection of HGPRT-deficient mutants, and 4) acceptance of a positive result if the induced mutant incidence is significantly above that of the simultaneous control and beyond the 98% confidence limitation of the mean of the population spontaneous mutant incidence. With this protocol, the ARL/HGPRT mutagenesis assay has activated representative members of the mycotoxin, amino azo dye, polycyclic aromatic hydrocarbon, and nitrosamine types of carcinogens, in addition to the aromatic amine compounds to be discussed.

In the ARL/HGPRT assay, 2-FAA is mutagenic (*31*), but a variation for enhancing the activity is provision of hepatocyte-mediated activation (*52*). In this variation of the assay, freshly dissociated HPC are cocultivated with the target ARL cells. The use of HPC has the advantage of providing the capability of carcinogen metabolism as documented in the HPC/DNA repair assay (table 1) plus the fact that the HPC cannot be subcultured upon replating to recover the ARL mutants. In this approach, HPC are inoculated with the ARL cells so that close contact for maximal exchange of metabolites is provided. Under conditions of exposure to 2-FAA that were not mutagenic to ARL cells alone, hepatocyte-medicated metabolites produced a mutagenic effect (table 3). Although this approach is successful with certain compounds and can also be used to generate bacterial mutagens (*12, 25*), its potential as a screening approach is limited because cell-to-cell transfer of activated metabolites is required. Therefore, in collaboration with Dr. Charles Tong, we have focused our attention on the delineation of the ability of ARL lines alone to activate mutagens.

A significant mutagenic response measured as 8-AG-resistant mutants was produced in the ARL-6 line following exposure to 10^{-6} M 2-FAA for 8 weeks as shown in table 4 (*31*). Using the current protocol, which involves a shorter duration of exposure and selection in TG, we confirmed that 2-FAA was mutagenic to ARL-6, but not to ARL-14, although a greater mutagenic response to aflatoxin B_1 occurred in this line than in ARL-6 (table 4). Thus in these two lines, the activation capability for two types of carcinogens appeared dissociated, which indicated a multiplicity of enzyme systems. Therefore, we believe these lines offer interesting possibilities for studies on the genetics and regulation of the activation systems involved.

The mutagenicity of other N-substituted aryl compounds was examined in the ARL-6 line, which was responsive to 2-FAA. Although *N*-OH-2-FAA was the most active on the line, 2-FA was more active than 2-FAA (table 5). Dybing et al. (*25*) also found that 2-FA was more mutagenic than 2-FAA in a system in which they used isolated rat hepatocytes to activate compounds to metabolites

TABLE 2.—*Mammalian cell mutagenicity of N-substituted aryl compounds*

Compound	Cell system	Locus	Dose	Mutant incidence (background)	Toxicity, %	References
2-FAA	V-74-4 Chinese hamster	HGPRT	20 μg/ml	$5/10^5$ ($5/10^5$)	0	(*41*)
N-OH-2-FAA			20 μg/ml	$50/10^5$ ($5/10^5$)	20	
N-AcO-2-FAA			4 μg/ml	$2{,}000/10^5$ ($5/10^5$)	90	
N-AcO-2-FAA	Human fibroblasts	HGPRT	6 μM	$400/10^6$ (NS)	95	(*42*)
N-AcO-*N*-acetyl-FA	V79 Chinese hamster	HGPRT	16 μM	$350/10^5$ ($1.4/10^5$)	99	(*43*)
N-AcO-*N*-myristoyl-FA			50 μM	$0.6/10^5$ ($1.4/10^5$)	11	
N-Myristoyloxy-*N*-2-FAA			50 μM	$15.5/10^5$ ($1.4/10^5$)	55	
N-Myristoyloxy-*N*-myristoyl-FA			25 μM	0 ($1.4/10^5$)	21	
2-FAA	L5178Y	Thymidine kinase	160 μg/ml	$98/10^6$ ($45/10^6$)	63	(*44*)
	+S9		75 μg/ml	$263/10^6$ ($43/10^6$)	78	
4-FAA			250 μg/ml	$104/10^6$ ($63/10^6$)	91	
	+S9		120 μg/ml	$83/10^6$ ($35/10^6$)	77	
N-AcO-*N*-acetyl-FA	C3H/10T1/2 C18 mouse fibroblast	Ouabain	15 μM	$9/10^5$ ($1/10^5$)	Not significant	(*45*)

TABLE 3.—*Hepatocyte-mediated mutagenesis of 2-FAA in the ARL/HGPRT assay*[a]

Cell system	Exposure	Colony forming efficiency, %	AG-resistant mutants/10^6 CFC
ARL-6	10^{-5} M	30	213
ARL-6 + HPC	"	36	322
HPC	"	0	0
ARL-6	None	26.2	168
ARL-6 + HPC	"	27.0	207
HPC	"	0	0

[a] Data are from (*52*). The assay conditions were different from those used in later work.

TABLE 4.—*Mutagenicity of 2-FAA and aflatoxin B_1 to ARL epithelial lines*

Cell system	AG-resistant mutants/10^6 CFC[a]			TG-resistant mutants/10^6 CFC		
	2-FAA	AFB_1	None	2-FAA[b]	AFB_1[c]	None
ARL-6	268	148	53–108	71 ± 9	41 ± 15	0–10
ARL-14	ND[d]	1,320	0–147	0 ± 0	120 ± 50	0–3

[a] Data are from (*31*); exposure to 10^{-6} M was for 8 wk.
[b] Exposure to 10^{-4} M was for 24 hr.
[c] Exposure to 10^{-6} M was for 24 hr.
[d] ND = not done.

TABLE 5.—*Relative mutagenicity of N-substituted aryl compounds to ARL 6 epithelial cell line*

Compound	Dose, M [a]	TG-resistant mutants/10^6 CFC
2-FA	10^{-3}	Toxic
	10^{-4}	57 ± 24
2-FAA	10^{-3}	77 ± 12
N-OH-2-FAA	10^{-4}	Toxic
	10^{-5}	108 ± 38
4-FAA	10^{-3}	4 ± 6
None	—	2 ± 5

[a] Exposure was for 24 hr.

that were mutagenic bacteria. Although 4-FAA was nonmutagenic to the ARL line (table 5), it did elicit DNA repair in hepatocytes (table 1).

To establish that the increased incidence of mutants following exposure to the arylamines was truly a result of metabolic activation of these compounds, we examined the effect of an inhibitor of metabolism. Paraoxon is a carboxyesterase-amidase inhibitor that delays the deacetylation of *N*-OH-2-FAA (*13*) and reduces its mutagenicity (*17, 53, 54*). Exposure of ARL-6 to both *N*-OH-2-FAA and paraoxon reduced the mutagenicity of *N*-OH-2-FAA for the line (table 6). Paraoxon produced no such effect on the mutagenicity of aflatoxin B_1 but rather increased its toxicity. Therefore, the inhibition of *N*-OH-2-FAA mutagenicity appears to have some specificity that is presuma-

TABLE 6.—*Effect of paraoxon on mutagenicity of* N-*OH-2-FAA to an ARL epithelial cell line*

N-OH-2-FAA[a]	Paraoxon	TG-resistant mutants/10^6 CFC
1×10^{-5}	None	108 ± 38
1×10^{-5}	10^{-4}	17 ± 15
1×10^{-5}	10^{-5}	32 ± 15
1×10^{-5}	10^{-6}	32 ± 16

[a] Exposure was for 24 hr.

bly due to inhibition of deacetylation. However, paraoxon clearly may have other effects.

CONCLUSIONS

The ARL lines have demonstrated both sensitivity and resistance to the mutagenic effects of N-substituted aryl compounds that require metabolic activation. Furthermore, the mutagenicity was modulated by an inhibitor of metabolism. Thus these lines represent a system in which the relationship of metabolic processes to ultimate mutagenic effects can be studied in intact cells. The use of subcellular fractions for the activation of N-substituted aryl compounds to mutagenic metabolites yielded results that do not correlate with in vivo carcinogenicity of the compounds (table 7). This is probably a consequence of the deficient metabolism performed by subcellular fractions, as discussed earlier. Whereas the metabolism in subcellular fractions may be appropriate for carcinogens that do not have major detoxification pathways (*11*), intact cell systems should be used whenever possible for the evaluation of tissue profiles of metabolism and capability for the production of mutagens.

The ARL lines also represent a potentially valuable resource for screening carcinogens. The two factors that determine the usefulness of a test in a screening battery are the metabolic parameters of the test system and the nature of the end point (*55*). Although most recommended screening batteries include mammalian mutagenesis assays, thus far no evidence indicates that mutagenesis in mammalian cells differs from that in bacteria. Specific study of this point is required, but if the end points in the two systems are similar, then a mammalian mutagenesis test is unnecessary in a battery that uses the more sensitive bacterial mutagenesis tests unless the metabolic parameters are different. Like the bacterial mutagenesis tests, all the mammalian mutagenesis assays except the ARL/HGPRT assay depend on an added enzyme preparation; therefore, they do not extend the metabolic scope of the battery. However, the ARL/HGPRT assay does provide the metabolism of intact cells. Earlier in this communication and elsewhere (*23, 24*) the artifactual nature of metabolism in enzyme preparations was discussed. A test such as the ARL/HGPRT that embodies intact cellular metabolism has the potential of providing results that would greatly strengthen those obtained in a test that depends on subcellular metabolism.

TABLE 7.—*Comparison of bacterial mutagenicity of N-substituted aryl compounds with the use of rodent liver subcellular fractions for activation and carcinogenesis in the liver*

Compound	Liver fraction	Mutagenicity: Revertants	Mutagenicity: Strains	Liver carcinogenicity	References
N-OH-2-FAA	Rat-1 nuclear	500	TA1538	+ + +	(*53*)
	Mouse-1 nuclear	2,500	TA1538	+	
2-FAA	Rat-S9	150	TA98	+ + +	(*54*)
	Rat-1S9	330	TA98	+	
	Mouse-S9	1,510	TA98	+	
	Mouse-1S9	6,630	TA98	?	
	Guinea pig-S9	2,280	TA98	—	
2-FA	Rat-1S9	85	TA98	+ + +	(*21*)
2-Aminophenanthrene	Rat-1S9	726	TA98	—	
2-FA	Rat-1S9	1	TA1535	+ + +	
2-Naphthylamine	Rat-1S9	15	TA1535	—	

REFERENCES

(*1*) GILLETTE JR, BRODIE BB, LADU BN: The oxidation of drugs by liver microsomes: On the role of TPNH and oxygen. J Pharmacol Exp Ther 119:532–540, 1957

(*2*) CONNEY AH, MILLER EC, MILLER JA: Substrate-induced synthesis and other properties of benzpyrene hydroxylase in rat liver. J Biol Chem 228:753–766, 1957

(*3*) BOOTH J, BOYLAND E: The biochemistry of aromatic amines. Enzymatic N-hydroxylation of arylamines and conversion of arylhydroxylamines into *o*-aminophenols. Biochem J 91:362–369, 1964

(*4*) IRVING CC: Enzymatic N-hydroxylation of the carcinogen 2-acetylaminofluorene and the metabolism of *N*-hydroxy-2-acetylaminofluorene-9-^{14}C in vitro. J Biol Chem 239:1589–1596, 1964

(*5*) GRAM TE, FOUTS JR: Time course differences in the metabolism of drugs by hepatic microsomes from rats, rabbits and mice. J Pharmacol Exp Ther 152:363–371, 1966

(*6*) GROVER PL, SIMS P: Enzyme-catalysed reactions of polycyclic hydrocarbons with deoxyribonucleic acids and protein in vitro. Biochem J 110:159–160, 1968

(*7*) GELBOIN HV: A microsome-dependent binding of benzo(*a*) pyrene to DNA. Cancer Res 29:1272–1276, 1969

(*8*) WIEBEL FJ, LEUTZ JC, DIAMOND L, et al: Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase in microsomes from rat tissues: Differential inhibition and stimulation by benzoflavones and organic solvents. Arch Biochem Biophys 144:78–86, 1971

(*9*) AMES BN, MCCANN J, YAMASAKI E: Methods for detecting carcinogens and mutagens with the *Salmonella*/microsome mutagenicity test. Mutat Res 31:347–364, 1975

(*10*) BARTSCH H, MALAVEILLE C, MONTESANO R: In vitro metabolism and microsome-mediated mutagenicity of diallcyl-nitrosomes in rats, hamsters, and mouse tissue. Cancer Res 35:644–651, 1975

(*11*) BRUSICK D, JAGANNATH DR, WEEKES V: The utilization of in vitro mutagenesis techniques to explain strain, age- and sex-related differences in dimethylnitrosamine tumor susceptibilities in mice. Mutat Res 41:51–60, 1976

(*12*) GREEN MH, BRIDGES BA, ROGERS AM, et al: Mutagen screening by a simplified bacterial fluctuation test: Use of microsomal preparations and whole liver cells for metabolic activation. Mutat Res 48:287–294, 1977

(*13*) IRVING CC: Enzymatic deacetylation of *N*-hydroxy-2-acetylaminofluorene by liver microsomes. Cancer Res 26:1390–1396, 1966

(*14*) KADLUBAR FF, MILLER JA, MILLER EC: Microsomal N-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Cancer Res 36:1196–1206, 1976

(*15*) LOWER GM, BRYAN GT: Enzymic deacetylation of carcinogenic arylacetamides by tissue microsomes of the dog and other species. J Toxicol Environ Health 1:421–432, 1976

(*16*) MEUNIER M, CHAUVEAU J: Binding of dimethylaminobenzene metabolites to DNA and proteins. 1. In vitro studies on a microsomal-dependent system. Int J Cancer 6:463, 1970

(*17*) SCHUT HA, WIRTH PJ, THORGEIRSSON SS: Mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system: The role of deacetylation by liver and kidney fractions from mouse and rat. Mol Pharmacol 14:682–692, 1978

(*18*) KAWAJIRI K, YONEKAWA H, HARA E, et al: Biochemical basis for the resistance of guinea pigs to carcinogenesis by 2-acetylaminofluorene. Biochem Biophys Res Commun 85:959, 1978

(*19*) WEEKS CE, ALLABEN WT, LOUIS SC, et al: Role of arylhydroxamic acid acyltransferase in the mutagenicity of *N*-hydroxy-*N*-2-fluorenylacetamide in *Salmonella typhimurium*. Cancer Res 38:613–618, 1978

(*20*) ANDREWS LS, HINSON JA, GILLETTE JR: Studies on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in the Ames–*Salmonella* mutagenesis test system. Biochem Pharmacol 27:2399–2408, 1978

(*21*) SCRIBNER JD, FISK SR, SCRIBNER NK: Mechanism of action of carcinogenic aromatic amines: An investigation using mutagenesis in bacteria. Chem Biol Interact 26:11–25, 1979

(*22*) POUPKO JM, HEARN WL, RADOMSKI JL: N-Glucuronidation of N-hydroxy aromatic amines: A mechanism for their transport and bladder-specific carcinogenicity. Toxicol Appl Pharmacol 50:479–484, 1979

(*23*) WILLIAMS GM: A comparison of in vivo and in vitro metabolic activation systems, *In* Critical Reviews in Toxicology: Strategies for Short-term Testing for Mutagens/Carcinogens. (Butterworth B, ed). West Palm Beach, Fla: CRC Press, 1979, pp 96–97

(*24*) SCHMELTZ I, TOSK J, WILLIAMS GM: Comparison of the metabolic profiles of benzo(*a*)pyrene obtained from primary cell cultures and subcellular fractions derived from normal and methylcholanthrene-induced rat liver. Cancer Lett 5:81–89, 1978

(*25*) DYBING E, SODERLUND E, HAUG LT, et al: Metabolism and activation of 2-acetylaminofluorene in isolated rat hepatocytes. Cancer Res 39:3268–3275, 1979

(*26*) TONELLI QJ, CUSTER RP, SOROF S: Transformation of cultured mouse mammary glands by aromatic amines and amides and their derivatives. Cancer Res 39:1784–1792, 1979

(*27*) WIEBKIN P, FRY JR, JONES CA, et al: The metabolism of biphenyl by isolated viable rat hepatocytes. Xenobiotica 6:725–743, 1976

(*28*) KING CM, TRAUB NR, CARDENNA RA, et al: Comparative adduct formation of 4-aminobiphenyl and 2-aminofluorene derivatives with macromolecules of isolated liver parenchymal cells. Cancer Res 2374–2381, 1976

(*29*) WILLIAMS GM, ELLIOT JM, WEISBURGER JH: Carcinoma after malignant conversion in vitro of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res 33:606–612, 1973

(*30*) WILLIAMS GM: The study of chemical carcinogenesis using cultured rat liver cells. *In* Gene Expression and Carcinogenesis in Cultured Liver (Gerschenson LE, Thompson EB, eds). New York: Academic Press, 1975, pp 480–487

(*31*) _____: The use of liver epithelial cultures for the study of chemical carcinogenesis. Am J Pathol 85:739–753, 1976

(*32*) _____: The detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res 37:1845–1851, 1977

(*33*) CASCIANO DA, FARR JA, OLDHAM JD, et al: 2-Acetylaminofluorene-induced unscheduled DNA synthesis in hepatocytes isolated from 3-methylcholanthrene-treated rats. Cancer Lett 5:173–178, 1978

(*34*) MICHALOPOULOUS G, SATTLER GL, O'CONNOR L, et al: Unscheduled DNA synthesis induced by procarcinogens in suspensions and primary cultures of hepatocytes on collagen membranes. Cancer Res 38:1866–1871, 1978

(*35*) YAGER JD, MILLER JA: DNA repair in primary cultures of rat hepatocytes. Cancer Res 38:4385–4394, 1978

(*36*) WILLIAMS GM: Further improvements in the hepatocytes DNA repair test for carcinogens: Detection of carcinogen biphenyl derivatives. Cancer Lett 4:69–75, 1978

(*37*) DEAN BJ, HODSON-WALKER G: An in vitro chromosome assay using cultured rat liver cells. Mutat Res 64:329–337, 1979

(*38*) PROBST GS, HILL LE: Chemically-induced DNA repair synthesis in primary rat hepatocytes: A correlation with bacterial mutagenicity. Ann NY Acad Sci 349:405–406, 1980

(*39*) WILLIAMS GM: Carcinogen-induced DNA repair in primary rat liver cell cultures; a possible screen for chemical carcinogens. Cancer Lett 1:231–235, 1976

(*40*) BRANDT WN, FLAMM WG, BERNHEIM NJ: The value of hydroxyurea in assessing repair synthesis of DNA in HeLa cells. Chem Biol Interact 5:327–339, 1972

(*41*) HUBERMAN E, DONOVAN PJ, DIPAOLO JA: Mutation and transformation of cultured mammalian cells by *N*-acetoxy-*N*-fluorenylacetamide. J Natl Cancer Inst 48: 837–840, 1972

(*42*) MAHER VM, WESSEL JE: Mutations to azaguanine resistance induced in cultured diploid human fibroblasts by the carcinogen *N*-acetoxy-2-acetylaminofluorene. Mutat Res 28:277–284, 1975

(*43*) KUROKI T, BARTSCH H: Mutagenicity of some N- and O-acyl derivatives of *N*-hydroxy-2-aminofluorene in V79 Chinese hamster cells. Cancer Lett 6:67–72, 1979

(*44*) CLIVE D, JOHNSON KO, SPECTOR JF, et al: Validation and characterization of the L51784/TK$^{+/-}$ mouse lymphoma mutagen assay system. Mutat Res 59:61–108, 1979

(*45*) LANDOLPH JF, HEIDELBERGER C: Chemical carcinogens produce mutations to ouabain resistance in transformable C3H/10T1/2 C18 mouse fibroblasts. Proc Natl Acad Sci USA 76:930–934, 1979

(*46*) WILLIAMS GM: Primary and long-term culture of adult rat liver epithelial cells. Methods Cell Biol 14:357–364, 1976

(*47*) SAN RH, LASPIA MF, SOIEFER AI, et al: A survey of growth in soft agar and cell surface properties as markers for transformation in adult rat liver epithelial-like cell cultures. Cancer Res 39:1026–1034, 1979

(*48*) SAN RH, SHIMADA T, MASLANSKY CJ, et al: Growth characteristics and enzyme activities as further markers for

transformation in adult rat liver epithelial-like cell cultures. Cancer Res 39:4441–4448, 1979

(*49*) Williams GM, Tong C, Berman JJ: Characterization of analog resistance and purine metabolism of adult rat liver epithelial cell 8-azaguanine-resistant mutants. Mutat Res 49:102–115, 1978

(*50*) Tong C, Williams GM: Induction of purine analog-resistant mutants in adult rat liver epithelial lines by metabolic activation-dependent and independent carcinogens. Mutat Res 58:339–352, 1978

(*51*) _____: Definition of conditions for the detection of genotoxic chemicals in the adult rat liver hypoxanthine-guanine phosphoribosyltransferase (ARL/HGRPT) mutagenesis assay. Mutat Res 74:1–9, 1980

(*52*) San RH, Williams GM: Rat heptatocyte primary cell culture-mediated mutagenesis of adult rat liver epithelial cells by procarcinogens. Proc Soc Exp Biol Med 156:534–538, 1977

(*53*) Sakai S, Reinhold CE, Wirth PJ, et al: Mechanism of in vitro mutagenic activation and covalent binding of *N*-hydroxy-2-acetylaminofluorene in isolated liver cell nuclei from rat and mouse. Cancer Res 38:2058–2067, 1978

(*54*) Okuno S, Takeishi K, Seno T: Differential effect of a microsomal deacetylase inhibitor on the mutagenicity in *Salmonella typhimurium* of 2-acetylaminofluorene by liver homogenates of guinea pig, mice and rats. Cancer Lett 6:1–5, 1979

(*55*) Williams GM: The detection of chemical mutagens/carcinogens by DNA repair and mutagenesis in liver cultures. *In* Chemical Mutagens, vol VI (DeSerres FJ, Hollaender A, eds). New York: Plenum Press, 1980, pp 61–79

Transformation of Hamster Embryo Cells by Aromatic Amines[1, 2]

R. J. Pienta and J. C. Kawalek [3]

ABSTRACT—A mammalian cell transformation system that required cryopreserved primary cultures of Syrian golden hamster embryo cells was used in the evaluation of the carcinogenic potential of 46 structurally related aromatic amines. The results generally correlated with those obtained with animal bioassay systems. However, several carcinogenic compounds required the addition of an exogenous metabolic activation system provided by hamster liver S9 homogenate enzymes or cultured hepatocytes to transform the hamster embryo cells.—Natl Cancer Inst Monogr 58: 243–251, 1981.

The extensive amount of data discussed at this Conference clearly shows that aromatic amines present a major class of industrial chemicals. Many of these are potent carcinogens in several species of animals and a number of them have been implicated as causative agents of cancer in man (*1, 2*). As new compounds are synthesized, it is important that those which pose a hazard to man be identified so that human exposure to these agents can be prevented or regulated. The task will become less difficult as more information is obtained on the biologic principles involved in the activation and detoxification of this class of carcinogens (*3*). Studies concerning the metabolism of well-known carcinogens can lead to some generalized conclusions regarding the metabolism and carcinogenic potential of new and yet untested compounds.

Previous speakers have described in great detail the in vivo and in vitro biotransformation of several N-substituted aryl compounds to their active metabolites, the interaction of these products with nucleic acids, and the expression of these adducts by carcinogenesis in vivo and mutagenesis in vitro.

Diverse chemicals have induced the malignant transformation of mammalian cells treated in vitro (*4–9*). A short-term, cell transformation, carcinogenesis bioassay with Syrian golden hamster embryo cells has been used in the evaluation of numerous aromatic amino compounds. Comparisons of the relationships of chemical structure and biologic activity among the several classes of aromatic amines will be presented.

Abbreviation: 2-FAA = *N*-2-fluorenylacetamide.

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MATERIALS AND METHODS

The carcinogenic potential of the various chemicals examined in this study was determined by means of the in vitro mammalian cell transformation system with the use of Syrian golden hamster embryo cells. Hamster embryo cells have been used extensively in model systems for the detection of the carcinogenic activity of diverse chemicals (*4, 8, 10, 11*). The use of cells cryopreserved in liquid nitrogen as a source of target cells was described in a clonal hamster cell assay (*8*). Batches of primary cultures were prepared from pooled littermates of 12- to 13-day-old embryos. The cultures were trypsinized and stored in glass-sealed ampuls in liquid nitrogen in aliquot samples of 2.5×10^6 cells each. Cells used for feeder cultures were prepared similarly but stored at 5×10^6 cells/ampul. Samples from each batch of pooled embryos were then tested for susceptibility to known carcinogens such as benzo[*a*]-pyrene or 3-methylcholanthrene. Susceptible cultures were used in routine bioassays. In a typical bioassay, 500 secondary passage cells were planted onto each 50-mm dish previously seeded with approximately $6–8 \times 10^4$ X-irradiated feeder cells. On the following day, we added the candidate chemicals. The cultures were incubated for 8 days, then fixed in absolute methanol, and stained with 10% Giemsa. Then we examined individual colonies under low magnification (× 7–40) for the presence of morphologically transformed colonies indicated by three-dimensional, random, criss-cross growth of cells not observed in normal colonies. Examples of a normal and transformed colony are illustrated in figure 1. Compounds that were considered carcinogenic from reports available at the time but which gave negative results in the cell transformation bioassay were retested with an exogenous system for metabolic activation, provided either by the $9{,}000 \times g$ supernatant fractions of hamster liver homogenates (S9) or cultured hamster hepatocytes (*10, 12*). The S9 homogenates were added with or immediately after the chemical. The medium was changed 4–18 hours later and replaced with chemical/S9-free medium. Cultures were incubated further for 8 days. Primary cultures of hepatocytes, prepared from the livers of adult hamsters (*10, 12*), were added to the cell cultures immediately before the addition of the test chemical; they remained for the duration of the bioassay. Each bioassay experiment included a known carcinogen as a positive control, as well as cultures treated with medium alone or the solvent used for preparing the stock cultures of chemicals being tested.

RESULTS AND DISCUSSION

We tested the capacity of 46 aromatic amines including some metabolic derivatives from various structural categories to induce morphologic transformation of the hamster cells. Of this number, 33 were considered carcinogenic from assays in animals; 8 were designated as noncarcinogenic. The carcinogenic potential of the remaining 5 compounds was unknown because tests in animals were unavailable or incomplete. For convenience, the compounds were categorized on the basis of their aromatic ring systems, i.e., substituted aminobenzenes, azo dyes, aminobiphenyls, 2-fluorenamine and metabolic derivatives, methylene-bridged aminobenzenes, nitrosamines, polycyclic aromatic amines, and heterocyclic and miscellaneous aromatic amines.

Substituted Aminobenzenes

The results with the 10 compounds (text-fig. 1) in this category are summarized in table 1; aniline and its chlorinated derivatives, *o*-chloroaniline and *p*-chloroaniline, were tested; only the latter compound gave positive results. At the time these chemicals were bioassayed in the hamster embryo cell transformation system, aniline was considered to be noncarcinogenic; therefore, it was not retested in the presence of exogenous metabolic activation. The carcinogenicity of aniline has recently been demonstrated in F344 rats (*13*).

The remaining 7 compounds were variously substituted phenylenediamines, some of which are components of hair dyes. The carcinogens, 2,4-toluenediamine and 2-nitro-*p*-phenylenediamine, transformed hamster cells, although 2,4-diaminoanisole did not. In a preliminary bioassay experiment, hamster cells were transformed by *m*-phenylenediamine, which recently has been shown to be noncarcinogenic in mice and rats (*1*). However, negative results were obtained when a purified sample was tested double blind, whereas another noncarcinogen, 4-nitro-*o*-phenylenediamine, gave positive results when tested similarly. Interestingly, these compounds also gave positive responses in the Ames *Salmonella* mutagenicity test (*14*). The compounds Hair Color Blue No. 1 and Hair Color Yellow No. 3, whose carcinogenicities are undetermined, failed to transform hamster cells in preliminary bioassays when tested at 0.05–50.0 μg/ml. Inasmuch as these and other compounds gave negative responses in the standard test, results should not be considered definitive until they have been retested in the presence of liver S9 preparations or cultured hepatocytes because the target cell cultures may lack sufficient enzymatic activity to metabolize those which may be carcinogens.

Azo Dyes

At the time these azo dyes, shown in text-figure 2 and table 2, were tested, 2-methyl-4-dimethylaminoazobenzene was considered noncarcinogenic and did not transform the

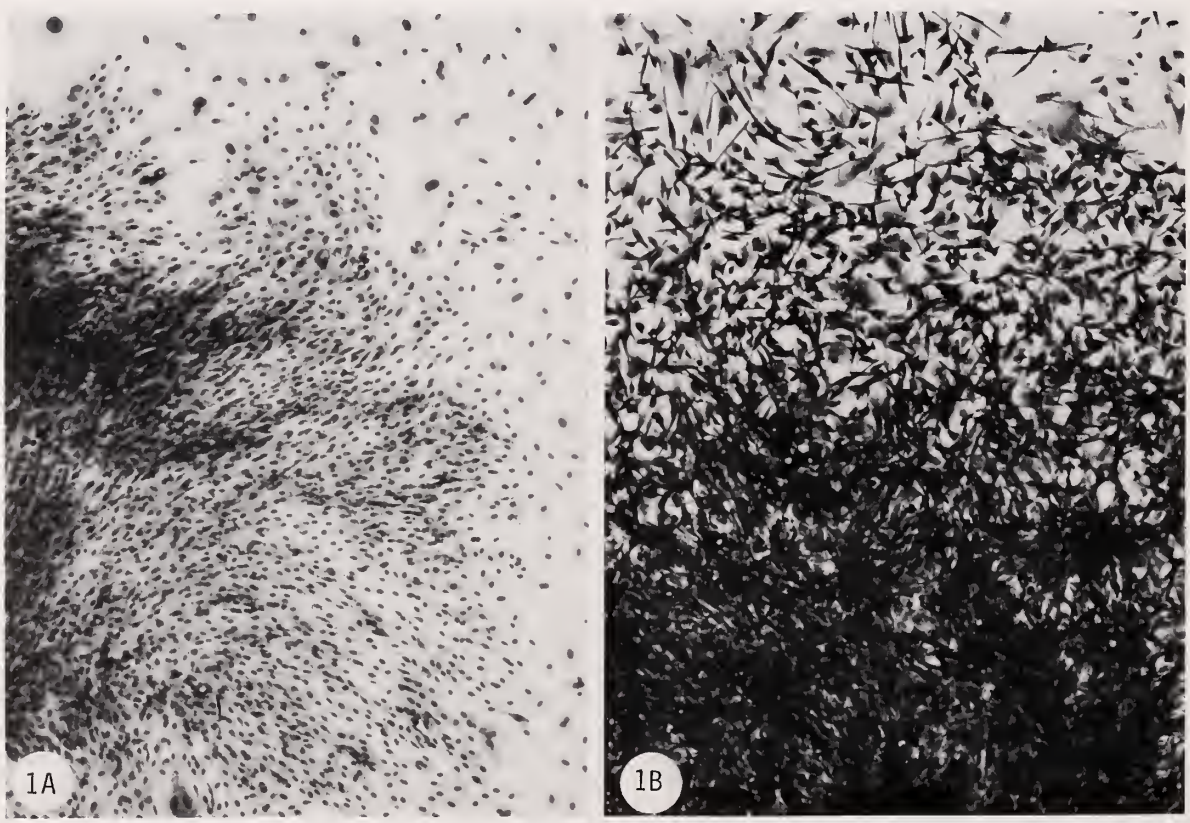
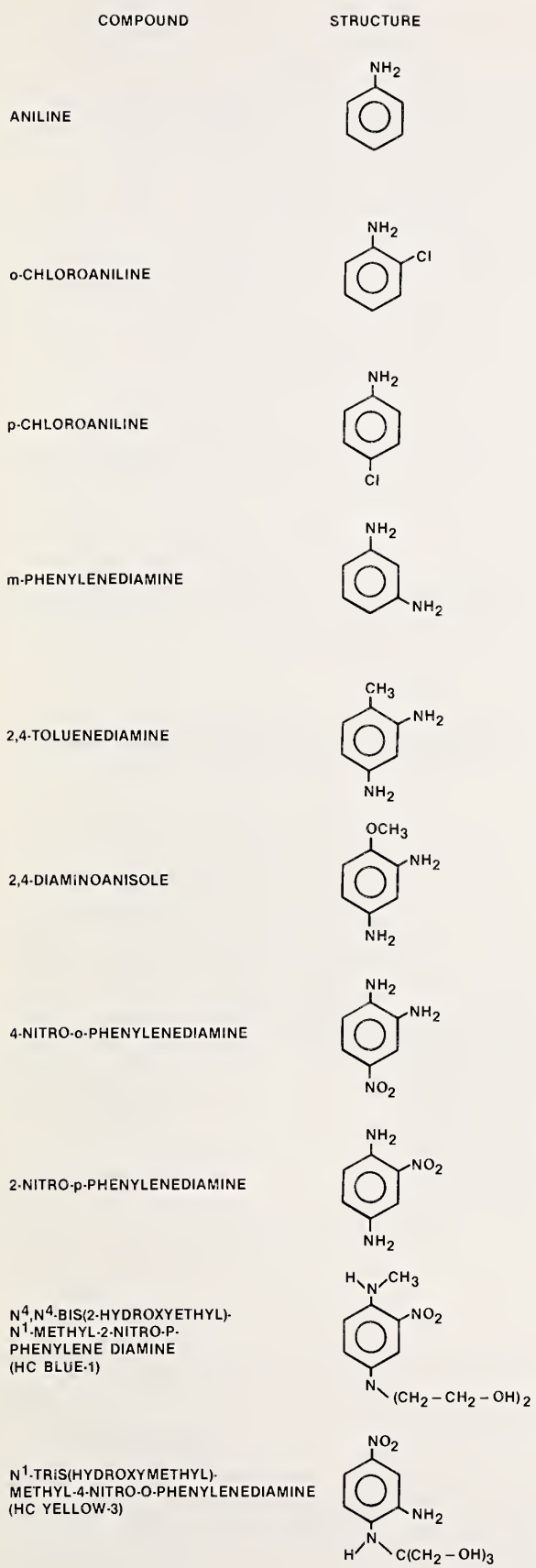


FIGURE 1.—Colonies of early passage of Syrian golden hamster embryo cells. A) Normal colony after 8 days in culture. Giemsa. × 40. B) Chemically induced, transformed colony after treatment for 8 days. Giemsa. × 40



TEXT-FIGURE 1.—Substituted amino benzenes.

TABLE 1.—*Substituted amino benzenes*

Compound	Carcinogenicity[a]	Transformation	Dose range tested, μg/ml	Transforming doses, μg/ml[b]
Aniline	C	–	0.01–10	—
o-Chloroaniline	NC	–	0.01–10	—
p-Chloroaniline	?	+	0.01–100	0.01, 1, 10, 100
m-Phenylenediamine	NC	+	0.05–50	0.05, 0.5, 5
		–[c]	0.1–20	—
2,4-Toluenediamine	C	+	0.05–50	0.5
		+[c]	0.63–10	2.5, 5
2,4-Diaminoanisole	C	–	0.05–50	—
4-Nitro-*o*-phenylenediamine	NC	+[c]	0.05–50	0.5, 5, 50
2-Nitro-*p*-phenylenediamine	C	+[c]	0.05–50	0.5, 5
N^4,N^4-Bis(2-hydroxyethyl)-N^1-methyl-2-nitro-*p*-phenylenediamine (Hair Color Blue No. 1)	?	–	0.05–50	—
N^1-Tris(hydroxymethyl)-methyl-4-nitro-*O*-phenylenediamine (Hair Color Yellow No. 3)	?	–	0.05–50	—

[a] C = carcinogen; NC = noncarcinogen; ? = undetermined.
[b] Dashes = no response, hence no transforming doses in tables 1–6.
[c] Purified sample was tested double blind.

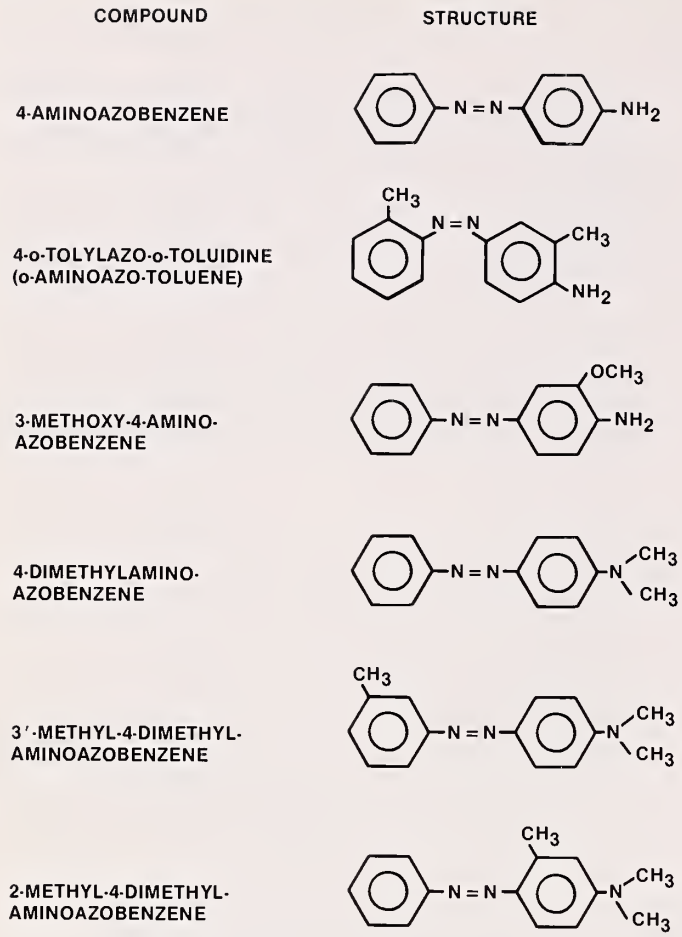
hamster embryo cells. However, more recently, the compound demonstrated weak hepatocarcinogenicity in weaning rats. Feeding the animals phenobarbital for up to 70 weeks could enhance the carcinogenicity (*15*). The other 5 carcinogens transformed hamster embryo cells under various test conditions. The compounds 4-*o*-tolylazo-*o*-toluidine, 3′-methyl-4-dimethylaminoazobenzene, and 4-dimethylaminoazobenzene gave positive results in the standard assay. However, 3-methoxy-4-aminoazobenzene was positive when hamster liver S9 was included in the test medium, and 4-aminoazobenzene gave a positive response in the presence of intact hamster hepatocytes. Neither compound was positive in the standard test in the absence of an added metabolic activation system.

Aminobiphenyls

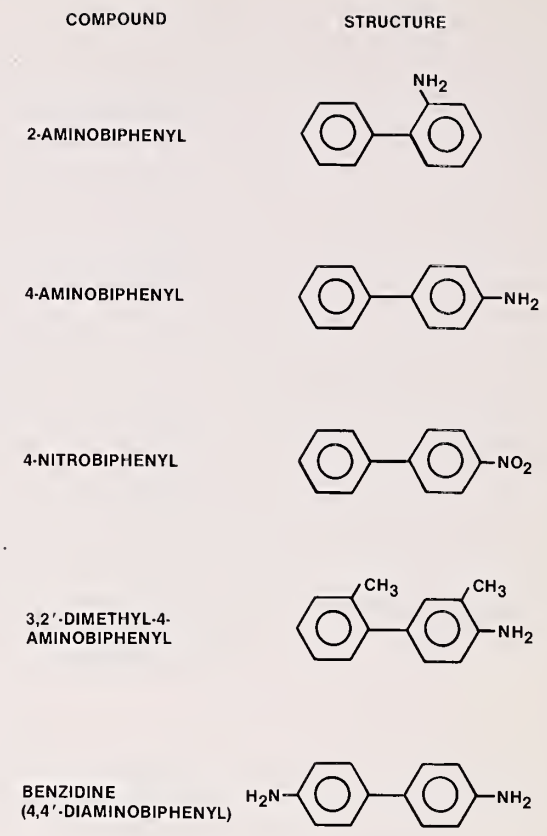
In this series, 2-aminobiphenyl, the only noncarcinogenic compound, did not induce cell transformation. The remaining 4 compounds, all well-known carcinogens, transformed hamster embryo cells under standard bioassay conditions (table 3, text-fig. 3).

2-Aminofluorene and Derivatives

The parent compound, 2-fluorenamine, transformed hamster cells, as did the N-hydroxy and nitro derivatives (table 4, text-fig. 4). However, 2-nitrofluorene required exogenous metabolism provided by either hamster liver S9



TEXT-FIGURE 2.—Azo dyes.



TEXT-FIGURE 3.—Aminobiphenyls.

TABLE 2.—*Azo dyes*

Compound	Carcinogenicity[a]	Transformation	Dose range tested, μg/ml	Transforming doses, μg/ml
4-Aminoazobenzene	C	+	3.2–32	32 [b]
		−	1–100	—
4-*o*-Tolylazo-*o*-toluidine (*o*-Aminoazo-toluene)	C	+	1–100	1.0
3-Methoxy-4-aminoazo-benzene	C	+	25, 50	25, 50 [c]
		−	1–100	—
4-Dimethylaminoazo-benzene	C	+	0.5–50	5
3'-Methyl-4-dimethyl-aminoazobenzene	C	+	1–100	10
2-Methyl-4-dimethyl-aminoazobenzene	C	−	1–100	—

[a] C = carcinogen; NC = noncarcinogen.
[b] Response was activated by cultured hamster hepatocytes.
[c] Response required activation by hamster liver S9.

or cultured hepatocytes. Positive responses were noted for 2-FAA and its hydroxy and acetoxy derivatives but not for *N*-4-FAA, the noncarcinogenic isomer of 2-FAA.

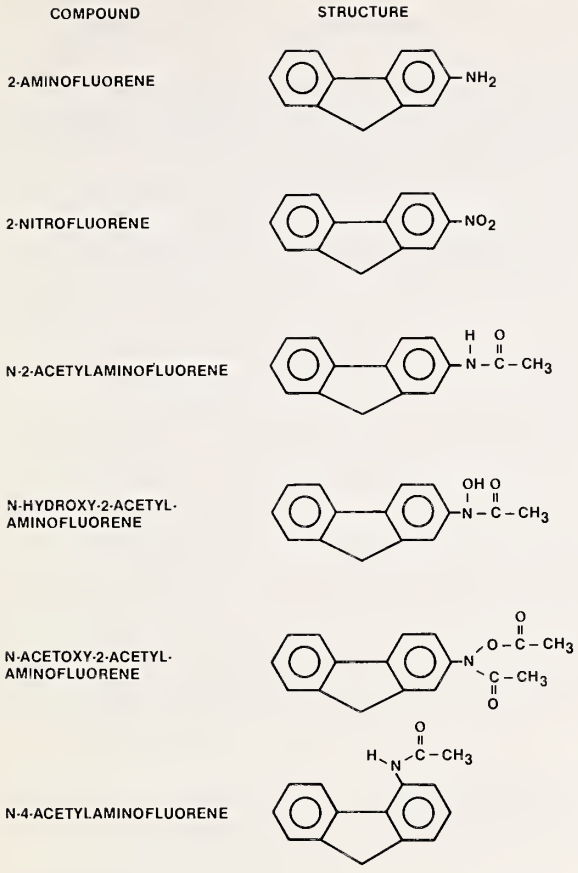
TABLE 3.—*Aminobiphenyls*

Compound	Carcinogenicity[a]	Transformation	Dose range tested, μg/ml	Transforming doses, μg/ml
2-Aminobiphenyl	NC	−	1–100	—
4-Aminobiphenyl	C	+	0.5–50	5, 50
4-Nitrobiphenyl	C	+	0.1–100	0.1, 1, 10
3,2'-Dimethyl-4-aminobiphenyl	C	+	1–100	0.1, 1, 10
Benzidine (4,4'-diaminobiphenyl)	C	+	0.5–50	0.05, 0.5, 5

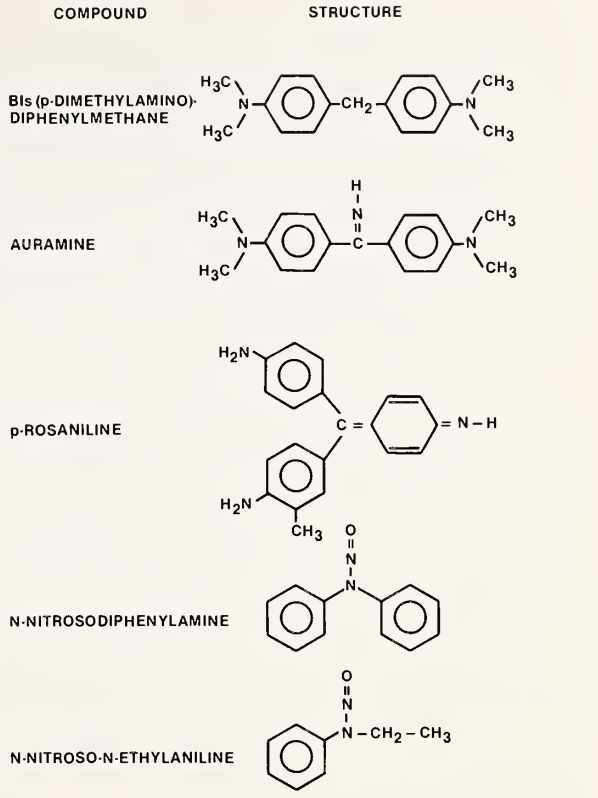
[a] C = carcinogen; NC = noncarcinogen.

Methylene-Bridged Aminobenzenes and Nitrosamines

The results summarized in table 5 show that the carcinogens bis(*p*-dimethylamino)diphenylmethane, auramine, and *p*-rosaniline (text-fig. 5) transformed hamster embryo cells, although hamster liver S9 was required for transformation by the latter 2 compounds. The aromatic nitrosamines, *N*-nitrosodiphenylamine and *N*-nitroso-*N*-ethylaniline, gave positive results in the standard cell transformation assay when tested at several doses. Although previously thought to be noncarcinogenic, *N*-nitrosodiphenylamine, when fed to rats, induced urinary bladder tumors (*16*). Because *N*-methyl-nitrosoaniline is a known



TEXT-FIGURE 4.—2-Fluorenamine and derivatives.



TEXT-FIGURE 5.—Methylene-bridged aminobenzenes and nitrosamines.

TABLE 4.—*Fluorenamine and derivatives*

Compound	Carcino-genic-ity[a]	Trans-forma-tion	Dose range tested, μg/ml	Trans-forming doses, μg/ml
2-Fluorenamine	C	+	0.1–100	1, 5, 10
2-Nitrofluorene	C	+	5, 10, 20	20[b]
		−	0.1–100	—
2-FAA	C	+	0.1–10	5, 10
N-hydroxy-2-FAA	C	+	0.1–10	1, 5, 10
N-acetoxy-2-FAA	C	+	1–10	5, 10
N-4-FAA	NC	−	0.5–50	—

[a] C = carcinogen; NC = noncarcinogen.
[b] Response required activation by cultured hamster hepatocytes or hamster liver S9.

TABLE 5.—*Methylene-bridged aminobenzenes and nitrosamines*

Compound	Carcino-genic-ity[a]	Trans-forma-tion	Dose range tested, μg/ml	Trans-forming doses, μg/ml
Bis(*p*-dimethylamino) diphenylmethane	C	+	0.1–100	1, 10
Auramine	C	+	1, 2	2[b]
		−	0.01–100	—
p-Rosaniline	C	+	1, 2	2[b]
		−	0.01–100	—
N-Nitrosodiphenylamine	C	+	0.63–63	6.3, 20
N-Nitroso-*N*-ethyl-aniline	?	+	0.2–200	0.2, 0.63, 6.3

[a] C = carcinogen; ? = undetermined.
[b] Response was activated by hamster liver S9.

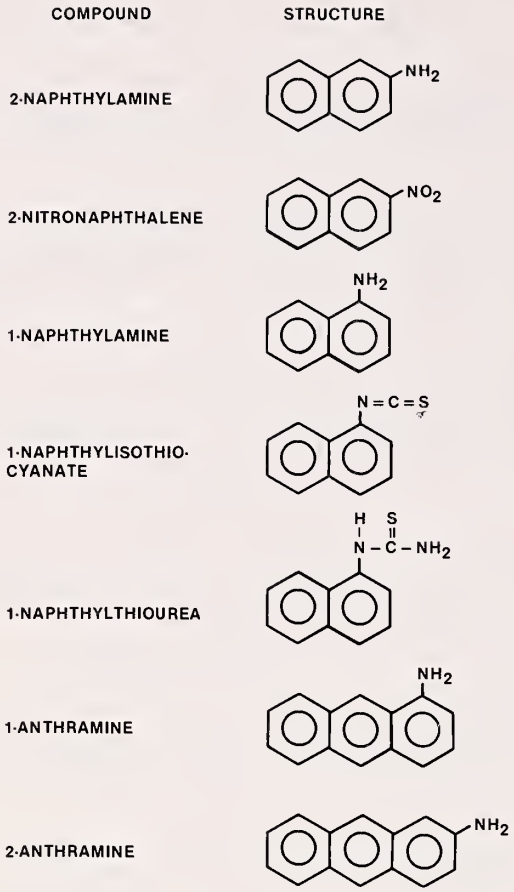
carcinogen (*17*), one should expect similar activity from the ethyl analog, *N*-nitroso-*N*-ethylaniline.

Polycyclic Aromatic Amines

In this series of compounds (table 6, text-fig. 6), the 1-amino(α) derivatives of both naphthalene and anthracene, i.e., 1-naphthylamine and 1-anthramine, respectively, gave negative results, whereas the carcinogenic 2-amino(β) derivatives induced cell transformation. The nitro-substituted carcinogen, 2-nitronaphthalene, also had positive results. α-Naphthylisothiocyanate, although a potent alkylating agent (*18*) but noncarcinogenic in animals, did not transform cells. 1-Naphthylthiourea, another alkylating agent (*19*) whose carcinogenicity is in question (*20*) due to contamination with 1- and 2-naphthylamines (*21*), gave positive results in the hamster cell transformation assay at every dose level tested (*22*).

Heterocyclic and Miscellaneous Aromatic Amines

Results with these 7 aromatic amines (table 7, text-fig. 7)



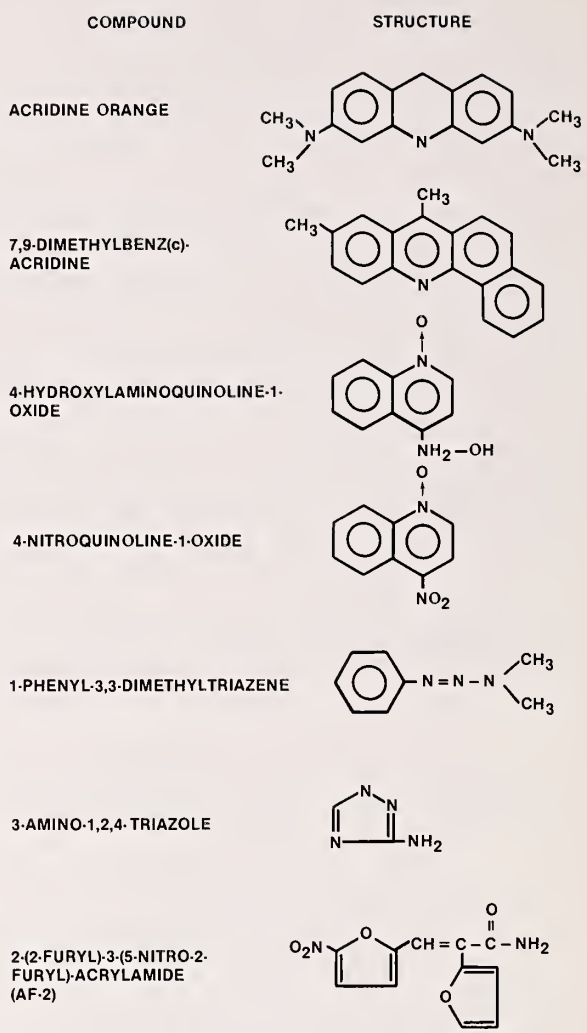
TEXT-FIGURE 6.—Polycyclic aromatic amines.

TABLE 6.—*Polycyclic aromatic amines*

Compound	Carcinogenicity[a]	Transformation	Dose range tested, μg/ml	Transforming doses, μg/ml
2-Naphthylamine	C	+	0.1–100	0.1, 1, 10
2-Nitronaphthalene	C	+	0.1–100	0.1, 1, 10
1-Naphthylamine	NC	–	0.1–100	—
1-Naphthylisothiocyanate	NC	–	0.1–100	—
1-Naphthylthiourea	?	+	1.6–25	1.6, 3.1, 6.3, 12.5, 25
1-Anthramine	NC	–	0.1–100	—
2-Anthramine	C	+	0.1–100	0.1, 1, 10

[a] C = carcinogen; NC = noncarcinogen; ? = undetermined.

show that all are carcinogenic and gave positive results in the cell transformation test. This group includes 2 acridine derivatives, 2 quinoline oxide derivatives, an aminotriazole, a substituted triazine, and a nitrofuran. The latter chemical, known as AF-2, was used extensively in Japan as a food preservative for almost 10 years before it was found to be mutagenic in short-term tests and carcinogenic in rodents.



TEXT-FIGURE 7.—Heterocyclic and miscellaneous aromatic amines.

TABLE 7.—*Heterocyclic and miscellaneous aromatic amines*

Compound	Carcinogenicity[a]	Transformation	Dose range tested, μg/ml	Transforming doses, μg/ml
Acridine orange	C	+	0.001–0.01	0.001
7,9-Dimethylbenz(*c*)acridine	C	+	0.1–10	0.1, 1, 10
4-Hydroxylaminoquinoline-1-oxide	C	+	0.0005–0.5	0.5
4-Nitroquinoline-1-oxide	C	+	0.0001–0.01	0.001, 0.01
1-Phenyl-3,3-dimethyltriazene	C	+	0.1–100	0.1
3-Amino-1,2,4-triazole	C	+	0.1–10	1, 10
2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2)	C	+	0.25–2.0	0.25, 0.5, 2

[a] C = carcinogen.

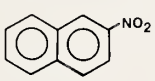
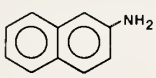
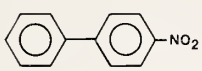
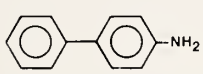
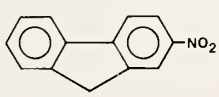
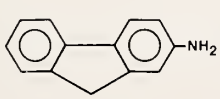
Need for Exogenous Metabolic Activation

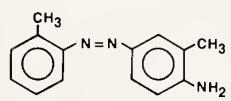
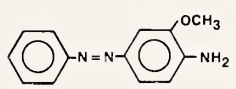
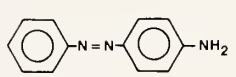
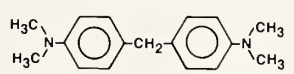
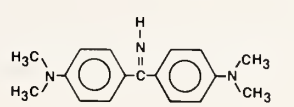
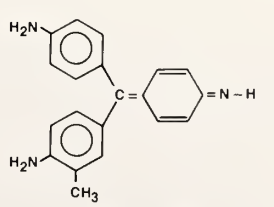
A prerequisite for the transformation of mammalian cells in culture is the ability of the cells to metabolize carcinogens to their active forms. Apparently, Syrian hamster embryo cells generally have this capacity as evidenced by the diverse classes of carcinogens that can induce transformation in this cell system (*4, 8, 10*). Nevertheless, some carcinogens did not transform hamster embryo cells, apparently because of an absence or insufficiency in the level of endogenous enzymes in occasional batches of the hamster embryo cells that were used.

With 2-FAA as the substrate, the rate of N-hydroxylation by hamster embryo cells was 90 times less than that observed with intact hamster hepatocytes (*23*). This low rate of N-hydroxylation may account for the inability of certain aromatic amines to transform hamster embryo cells into malignant cells. Hamster hepatocytes possessed higher basal and induced levels of aryl hydrocarbon hydroxylase activity than did hamster embryo cells (*12*); also, the ability of hamster embryo cells to be transformed by polycyclic aromatic hydrocarbons correlated directly with the inducibility of this enzyme in the cells (*24*).

This problem of low endogenous activity in hamster embryo cells can be circumvented by the incorporation of a source of exogenous metabolic activation. Enzyme preparations from rodent liver homogenates have been used successfully in bacterial (*25*) as well as mammalian cell (*26*) mutagenesis systems. More recently, hamster liver S9 preparations or primary cultures of hepatocytes from adult Syrian golden hamsters have been used to provide metabolic activation in the hamster embryo cell transformation system (*10, 12*). Transformation was observed only when hepatocytes were added to cultures treated with diethylnitrosamine, 2-nitrofluorene, or 4-aminoazobenzene (*12*). In the present study, the requirement for an exogenous metabolic activation system was observed with 2-nitrofluorene, 4-aminoazobenzene, 3-methoxy-4-aminoazobenzene, auramine, and *p*-rosaniline.

The results in this report demonstrate that the effectiveness of the standard bioassay can be improved by the addition of an exogenous metabolic activation system to eliminate apparent false-negative responses. However, paradoxical results are obtained with some chemicals that essentially undergo similar metabolic reactions when activated to reactive products. These are illustrated in text-figure 8. For example, 4-nitrobiphenyl and 2-nitronaphthalene as well as their amine derivatives, i.e., 4-aminobiphenyl and 2-naphthylamine, were capable of transforming hamster embryo cell without the addition of

COMPOUND	STRUCTURE	REQUIRES EXOGENOUS METABOLIC ACTIVATION
2-NITRONAPHTHALENE		–
2-NAPHTHYLAMINE		–
4-NITRO-BIPHENYL		–
4-AMINOBIPHENYL		–
2-NITROFLUORENE		+
2-AMINOFLUORENE		–

COMPOUND	STRUCTURE	REQUIRES EXOGENOUS METABOLIC ACTIVATION
4-o-TOLYLAZO-o-TOLUIDINE (o-AMINOAZO-TOLUENE)		–
3-METHOXY-4-AMINO-AZOBENZENE		+
4-AMINOAZOBENZENE		+
Bis(p-DIMETHYLAMINO)-DIPHENYLMETHANE		–
AURAMINE		+
p-ROSANILINE		+

TEXT-FIGURE 8.—Need for exogenous metabolic activation by structurally related compounds.

exogenous metabolic activation. On the other hand, 2-nitrofluorene gave a positive response only in the presence of either hamster liver S9 or intact hepatocytes, but the parent amine, 2-fluorenamine, did not require these additional sources of metabolic activity. Similarly, 4-aminoazobenzene and 3-methoxy-4-aminoazobenzene required the exogenous activating systems, but 4-*o*-tolylazo-*o*-toluidine, similar in structure, gave positive transformation results without them.

Finally, *p*-rosaniline and auramine required an exogenous activation system for transformation. These compounds are structurally related to bis(*p*-dimethylamino) diphenylmethane which transformed hamster embryo cells in the standard assay without the addition of either hamster S9 or hepatocytes.

SUMMARY AND CONCLUSIONS

This study demonstrates the feasibility of one's using a hamster embryo cell transformation system that requires cryopreserved primary cultures of cells as a source of target cells as a tool for screening the carcinogenic potential of aromatic amines. Excellent correlation with reported activity in animal tests was obtained in a standard bioassay when 46 aryl amines were tested. However, several required the addition of an exogenous metabolic activation system provided by either hamster liver S9 homogenate enzymes or cultured hepatocytes. Of 8 purportedly noncarcinogenic compounds, 2 of them, 2-nitro-*o*-phenylenediamine and *m*-phenylenediamine, induced morphologic transformation of hamster embryo cells. However, when a purified sample was retested, the latter compounds have negative results. Three compounds, i.e., aniline, 2,4-diaminoanisole, and 2-methyl-4-dimethylaminoazobenzene, originally designated as noncarcinogens at the time they were bioassayed in cell culture but recently designated as carcinogens, gave negative results. These negative in vitro results should be considered with caution because the compounds were not retested in the presence of an added metabolic activation system. The carcinogenicity of 3 compounds is still to be determined, but all gave positive responses in the cell transformation system; these were *p*-chloroaniline, currently under bioassay in animals, *N*-nitroso-*N*-ethylaniline, which is the ethyl analog of *N*-nitroso-*N*-methylaniline, and 1-naphthylthiourea, which is both a potent alkylating agent and a rodenticide that causes pulmonary edema in rats. Although it is supposedly contaminated with 1- and 2-aminonaphthalenes, carcinogenicity in animal bioassays was not detected (*20*) and was possibly obscured by the high toxicity of 1-naphthylthiourea.

REFERENCES

(*1*) Weisburger EK, Russfield AB, Homburger F, et al: Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. J Environ Pathol Toxicol 2:325–356, 1978

(*2*) Patrianakas C, Hoffmann D: Chemical studies in tobacco smoke. LXIV. On the analysis of aromatic amines in cigarette smoke. J Anal Toxicol 3:150–154, 1979

(*3*) Irving CC: Species and tissue variations in the metabolic activation of aromatic amines. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 211–227

(*4*) DiPaolo JA, Nelson RL, Donovan PJ: In vitro transformation of Syrian hamster embryo cells by diverse chemical carcinogens. Nature 235:278–280, 1972

(*5*) DiPaolo JA, Casto BC: Chemical carcinogenesis. *In* Recent Advances in Cancer Research, Cell Biology, and Tumor Virology (Gallo RC, ed). Cleveland: CRC Press, 1977, pp 17–47

(*6*) Heidelberger C: Chemical oncogenesis in culture. Adv Cancer Res 18:317–366, 1973

(*7*) Mishra NK, DiMayorca G: In vitro malignant transformation of cells by chemical carcinogens. Biochim Biophys Acta 355:205–219, 1974

(*8*) Pienta RJ, Poiley JA, Lebherz WB III: Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable in vitro bioassay for identifying diverse carcinogens. Int J Cancer 19:642–655, 1977

(*9*) Tonelli QJ, Custer RP, Sorof S: Transformation of cultured mouse mammary glands by aromatic amines and amides and their derivatives. Cancer Res 39:1784–1792, 1979

(*10*) Pienta RJ: A hamster cell model system for identifying carcinogens. *In* Carcinogens: Identification and Mechanisms of Action (Griffin AC, Shaw CR, eds). New York: Raven Press, 1979, pp 121–141

(*11*) Berwald Y, Sachs L: In vitro transformation with chemical carcinogens. Nature 200:1182–1184, 1963

(*12*) Poiley JA, Raineri R, Pienta RJ: Use of hamster hepatocytes to metabolize carcinogens in an in vitro bioassay. J Natl Cancer Inst 63:519–524, 1979

(*13*) Division of Cancer Cause and Prevention, National Cancer Institute: Bioassay of aniline hydrochloride for possible carcinogenicity. Carcinogenesis Technical Report Ser. No. 130, CAS No. 142-04-1, DHEW Publ No. (NIH) 78-1385, Springfield, Va.: Natl Tech Inform Serv, 1978

(*14*) Ames BN, Kammen HO, Yamasaki E: Hair dyes are mutagenic: Identification of a variety of mutagenic agents. Proc Natl Acad Sci USA 72:2423–2427, 1975

(*15*) Kitagawa T, Pitot HC, Miller EC, et al: Promotion by dietary phenobarbital of hepatocarcinogenesis by 2-methyl-*N,N*-dimethyl-4-aminoazobenzene in the rat. Cancer Res 39:112–115, 1979

(*16*) Cardy RH, Lijinsky W, Hildebrandt, PK: Neoplastic and nonneoplastic urinary bladder lesions induced in Fischer 344 rats and B6C3F$_1$ hybrid mice by *N*-nitrosodiphenylamine. Ecotoxicol Environ Safety 3:29–35, 1979

(*17*) Goodall CM, Lijinsky W, Tomatis L, et al: Toxicity and oncogenicity of nitrosomethylaniline and nitrosomethylcyclohexylamine. Toxicol Appl Pharmacol 17:426–432, 1970

(*18*) El-Hawari AM, Plaa GL: α-Naphthylisothiocyanate (ANIT) hepatotoxicity and irreversible binding to rat liver microsomes. Biochem Pharmacol 26:1857–1866, 1977

(*19*) Boyd MR, Neal RA: Studies on the mechanism of toxicity and of development of tolerance to the pulmonary toxin, α-naphthylthiourea (ANTU). Drug Metab Dispos 4: 314–322, 1976

(*20*) National Cancer Institute: Survey of compounds which have been tested for carcinogenic activity. DHEW Publ No. (NIH) 75, PHS Publ No. 149. Washington, D.C.: U.S. Govt Print Off, 1972–73

(*21*) Orzelick R: Research finds aromatic amines exposure cause of increased number of tumors. Int J Health Safety 44:46–47, 1975

(*22*) Kawalek JC, Andrews AW, Pienta RJ: 1-Naphthylthiourea: A mutagenic rodenticide that transforms hamster embryo cells. Mol Pharmacol 15:678–684, 1979

(*23*) Raineri R, Poiley JA, Ernst MK, et al: A high pressure, liquid chromatography procedure for the separation of metabolites of 2-acetylaminofluorene from cells in culture. J Liq Chromatog 1:457–467, 1978

(*24*) Benedict WF, Gielen JE, Nebert DW: Polycyclic hydrocarbon-produced toxicity, transformation, and chromosomal aberrations as a function of aryl hydrocarbon hydroxylase activity in cell cultures. Int J Cancer 9: 435–451, 1972

(*25*) Ames BN, Durson WE, Yamasaki E, et al: Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 70:2281–2285, 1973

(*26*) Kuroki T, Drevon C, Montesano R: Microsome-mediated mutagenesis in V79 Chinese hamster cells by various nitrosamines. Cancer Res 37:1044–1050, 1977

Discussion VI [1]

D. J. Brusick: Does anyone have questions on the papers just presented?

J. Felton: I want to make some general comments about short-term assays. Should we use *Salmonella typhimurium* or mammalian cells? I see no argument there at all. What I see is that the use of short-term assays depends on what your question is. If you are asking: Do we have an active intermediate that is going to cause genetic damage? Why not use *Salmonella?* The answer is simple; Dr. Thorgeirsson showed us that such studies are possible and worth doing.

If you form an active intermediate, what happens? Is the particular cell in question sensitive? This question on sensitivity has been raised frequently during this Symposium regardless of the experimental animal or tissue used. How do you answer that question when one species and one type of cells are used? I believe that if you want to know what is cell sensitive and you can predict carcinogenicity, it would be advisable to set up cell lines in 6 species and 10 tissues. Of course, this all represents differences in repair or in transport once you get to the cell, or differences in how histones interact with particular genes that are turned on, etc.

G. Williams: Excuse me, Dr. Felton, I think you have gravely misunderstood the import of my talk. At no time did I suggest that mammalian cells would replace bacterial mutagenesis assays in a screening battery. In fact, I have repeatedly written and stated that bacterial mutagenesis assays must be *part* of the screening battery. I think that everyone working in the field now generally recognizes the need for a battery of tests, so no one is saying that one test should be replaced by another.

R. Pienta: Could I make one comment about activation? I mentioned that, in this hamster cell system, you can use either a hamster S-9 or a hepatocyte preparation. Another system might require an intact animal to metabolize this material. Then you would study the urine to see whether you have active metabolites. Commoner and Ames and their respective groups have shown that, if you feed rats 2-FAA and do the respective urine determinations, you can see an induced number of revertants in the Ames' system. We have fed 2-FAA to rats and found that 24- and 48-hour samples of that material also induces transformation. More recently, we were studying methapyrilene, which happened to be one of the fashionable compounds to work on because it is one of the over-the-counter sleep inducers. This was shown by Dr. Lijinsky to be a potent carcinogen in rats. When we tested it in the hamster system in the presence of hepatocytes, or S-9, or cells alone, it was always negative. However, if we took the urine from rats fed methapyrilene, we did, in fact, see transformation with 2 or 4% urine placed directly onto the cells without deconjugation by glucuronidase or other enzymes. That method can also be used. We are investigating the feasibility of using that modification to screen or flag chemicals that might be carcinogenic in animals.

Brusick: Does anybody have any questions?

Unidentified participant: I am confused about the terminology we are using here. We have transformed cells, we have mutants, we have cancer cells. I understood that the cancer cell is a mutant. It is also a transformed cell, although not all transformed cells are cancer cells. Transformation can be either beneficial or destructive; so is mutagenicity. Carcinogenesis is just an example of the destructive type of transformation.

Now, in Dr. Pienta's table 7, there is a column for carcinogenicity and one for transformation. I can understand a positive transformation and a negative carcinogenesis, but what I cannot understand is a positive carcinogenesis and a negative transformation.

I would like to have someone clarify this for me, unless there is a separate set of criteria for carcinogenesis and transformation.

Pienta: In the jargon of people who work with mammalian cells, transformation usually means a morphologic change in the cells different from that found in normal cells. It also means that these cells have acquired characteristics that make them no longer normal, so that if they are injected into a susceptible host, they will form tumors. That is the context in which transformation is used.

When we observe carcinogenicity and fail to observe transformation, we mean we did not see this morphologic change that is characteristic of malignant change. Therefore, for that particular incident, the cell system failed to detect that chemical as a carcinogen. It is all the same.

Brusick: One last comment. You may be referring, though I am not certain, to microbial transformation, which is a different process. The terminology is used, and it is exactly the same term, but it is not the same kind of process as cellular transformation.

Williams: In the context of malignant transformation of mammalian cells, there is an interesting phenomenon that has, I think, intrigued a lot of us for a few years now. In the available fibroblast systems in which both transformation and mutagenesis can be studied, generally speaking, the rates of transformation are much higher than the rates of mutagenesis. Sometimes, they are up to 1,000-fold higher for transformation than for mutagenesis. About the closest I have ever seen them come is tenfold higher for transformation.

Abbreviation: 2-FAA = *N*-2-fluorenylacetamide.

[1] Conducted at the International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Rockville, Maryland, November 7-9, 1979.

This makes it difficult to explain transformation on a genetic basis because you would expect, if transformation were a genetic event that was only one of many mutations possible in the cell, that a higher incidence of mutations would have to be induced so that transformation could occur.

We have the liver epithelial lines that I described for the mutagenesis study. We also completed a large survey of 14 objective quantifiable markers for transformation in these lines. We identified 3 of them that are highly reliable: growth in soft agar, reappearance of gamma-glutamyl transpeptidase activity, and the ability to form colonies in low calcium medium. These are all linked closely with cancer, as measured by inoculation of the cells into syngeneic animals as well as nude mice.

In the preliminary studies, we started to determine the incidence of mutation versus transformation in this system. We found that mutagenicity can be induced at a much higher level than transformation, exactly the reverse of the fibroblast systems.

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